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Award Number: W81XWH-04-1-0893

TITLE: High Throughput Screen to Identify Novel Drugs that Inhibit Prostate Cancer Metastasis

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REPORT DATE: October 2007

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-10-2007	Final	20 Sep 2004 – 19 Sep 2007

4. TITLE AND SUBTITLE

High Throughput Screen to Identify Novel Drugs that Inhibit Prostate Cancer Metastasis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0893

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Irwin H. Gelman, Ph.D.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Roswell Park Cancer Institute
Buffalo, NY 14263

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
Original contains colored plates: ALL DTIC reproductions will be in black and white.

14. ABSTRACT

We have proposed to developed indicator cell lines that would allow for the high throughput screening (HTS) for compounds that potentially inhibit prostate cancer (CaP) metastasis. The cell lines are based on stably expressing a construct containing the promoter of SSeCKS/ gravin/AKAP12- a metastasis-suppressor gene downregulated in CaP progression- linked to a green fluorescence protein (GFP), plus a control reporter, in metastatic CaP cells, and then screening for compounds that induce GFP. We also proposed to characterize the pathways controlling SSeCKS expression in CaP progression. UPDATE: Our data indicate that SSeCKS re-expression can be induced in CaP cell lines using inhibitors of histone deacetylation (TSA) but not by inhibitors of methylation (5-aza-C). We have now produced stable indicator C4-2 cells with GFP expression inducible by TSA and by retinoids. We have also characterized the cis- and trans-acting factors of the human SSeCKSα promoter required for transcriptional suppression in CaP cells.

15. SUBJECT TERMS
Drug discovery for prostate cancer using a cell-based assay. Prostate cancer, metastasis, drug discovery, SSeCKS/Gravin, HTS, tumor suppressor gene, gene regulation.

16. SECURITY CLASSIFICATION OF:				18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	33	19b. TELEPHONE NUMBER *(include area code)*

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Introduction

We have proposed to construct indicator prostate cancer (CaP) cell lines that could be used to identify novel drugs that could inhibit parameters of oncogenic and metastatic growth. The cell lines are based on the stable expression of the promoter from the SSeCKS/gravin/AKAP12 metastasis-suppressor fused to a green fluorescent protein (GFP) reporter, plus a control reporter plasmid. In a high throughput screen (HTS), compounds that induce GFP expression but have no major effect on the control reporter would be identified for further analysis as potential inhibitors of CaP progression. A second major aim of our study is to use the SSeCKS/gravin/AKAP12α promoter to characterize the signaling pathways as well as the *cis*- and *trans*-acting mechanisms leading to transcriptional downregulation in CaP cells. This analysis included a determination whether CaP-specific gene silencing involves hypermethylation of CpG islands in the SSeCKS promoters or changes in chromatin acetylation.

Body

Production and testing of SSeCKS-promoter/CaP indicator lines. Our first task (based on Task 1 in the Statement of Work) was to produce CaP lines stably expressing the human SSeCKS/gravinα promoter-GFP, plus a control reporter (described in Task 1 in the Statement of Work). The promoter was cloned into the pEGFP vector and in transient expression assays, this construct was shown to express high levels of green fluorescence in untransformed cells (murine NIH3T3 and human P69SV40T prostate epithelial) yet low GFP levels in LNCaP and C4-2 CaP cells. Instead of the originally intended control reporter (SEAP), we chose to use an RFP (red fluorescent protein) reporter (Clontech) that we fused to the TK promoter (Fig. 1). In transient assays, almost equal RFP expression levels were detected in all untransformed and CaP cell lines (data not shown). We then stably transfected P69, LNCaP and C4-2 cells with both plasmids, selected for neo^R colonies, and then FACS sorted pooled colonies for the desired phenotype: C4-2 and LNCaP cells were selected for low GFP, high RFP; P69 cell were selected for high GFP and RFP. 5 individual clones from each were expanded.

In order to fulfill the requirements of Task 4 (characterization of signaling pathways and transcription factors involved in SSeCKS transcriptional silencing in CaP cells), we then tested a panel of signal transduction inhibitors, differentiating agents and transcriptional deregulators for their ability to re-induced GFP and endogenous SSeCKS expression in the CaP cells while having minimal effect on RFP expression in either the CaP or P69 cells. Prior to this analysis, we first developed probes to detect changes in the levels of endogenous human SSeCKSα and β mRNA and protein isoforms. These included isoform-specific PCR primer sets (Fig. 2; was Fig. 3 in the 2005 update report), and from previous studies, polyclonal antibodies (Ab) that detected both α and β protein isoforms (3) as well as an Ab that detected only the α isoform (2).

Although data from other groups had shown cases where SSeCKS expression was suppressed by either promoter hypermethylation (1) or histone deacetylation (4), our data indicated that treatment of CaP cells

with methylation inhibitors (5-aza-C or deoxy-5-aza-C) had no effect on transcript levels where the histone deacetylase inhibitor, TSA, derepressed SSeCKS transcript levels roughly 6- to 10-fold (although, still significantly lower than untreated P69 cell levels). We then tested a panel of pathway inhibitory drugs to help define which pathways are responsible for SSeCKS downregulation in CaP and v-Src cells.

Interestingly, *endogenous* SSeCKS expression was induced by TSA and by inhibitors of p38, PI3K (Fig. 3), Src or MEK (not shown), but only in the absence of serum. Given the previous finding that SSeCKS transcription is serum-responsive (5), our current data suggest that serum increases the background signal of SSeCKS mRNA, i.e.- masks the derepressive effects of the inhibitors. In contrast, the exogenous SSeCKS/GFP was not induced by inhibitors of Src, MEK, p38, PI3K, or by TSA, as shown by FACS analysis for GFP expression (Fig. 4). However, the differentiating agents, atRA acid (Fig. 4) or calcitriol (not shown), did induce GFP expression. These FACS-based experiments were performed in the presence of serum. Indeed, Fig. 5 shows that TSA failed to induce endogenous SSeCKS expression in other human CaP lines grown in the presence of serum. Therefore, we are performing these same experiments in the absence of serum to determine if these inhibitory/differentiation compounds have greater derepressive effects on the exogenous promoter. One confounding result was that exogenous SSeCKS promoter expression (GFP) could be induced only 2.5- to 3-fold by treatment for three days with either TSA or atRA in the C4-2 clones. We tried to increase this level with either single pathways inhibitory drugs, such as the Src inhibitors, SKI-606 (not shown) or KX2-391 (Fig. 4), the MEK inhibitor, U0126, or the PI3K inhibitor, LY294002, or with combinations of these drugs with TSA, atRA and 5-deoxy-azaC. However, in FACS analyses, none of these drug combinations increased expression from the exogenous SSeCKS promoter sufficiently (Fig. 4); performing these experiments in the absence of serum had little effect (not shown). Thus, these cells would not be good candidates in a HTS for novel drugs since 1.5-fold increases by novel compounds would not be statistically significant.

More recently, we produced DU145 reporter cells with the αSSeCKS promoter/GFP construct described above. Unfortunately, these cells showed similar problems, namely that exogenous SSeCKS levels could not be induced more than 3-fold with TSA in the absence of serum, thereby making them poor candidates for HTS.

Continuing with Task 4 experiments, we addressed whether the endogenous human or mouse SSeCKS promoters could be induced by either synthetic testosterone (R1881) or by hypoxic conditions ($CoCl_2$). Fig. 6 shows that human SSeCKS can be induced by R1881, especially in the absence of serum, in keeping with previous data that it is an androgen-inducible gene (6). Fig. 6 also shows that in the absence of serum, SSeCKS is not induced under hypoxic conditions in both LNCaP NIH3T3 cells. This finding is in keeping with previous data showing that SSeCKS expression is not altered by hypoxia but is induced by the transition from hypoxia to normoxia (7).

In response to Task 3 of the Statement of Work, we analyzed the cis- and trans-acting factors that control SSeCKS promoter expression in CaP and 3T3/v-Src cells. We reasoned that common, if not overlapping control mechanisms and factors would be involved between the Src- and CaP-mediated SSeCKS downregulation. We previously identified minimal promoter fragment of -106 to +35 as encoding the CaP- and Src-responsive sequences in

transient transfection, luciferase-reporter assays. Using EMSA and ChIP assays, we previously showed that this promoter fragment encodes an upstream E-box that binds USF1 and a downstream GC-box that binds both Sp1 and Sp3. Interestingly, even though both boxes are required for the CaP- and v-Src-associated SSeCKS downregulation, binding to the downstream Sp1/Sp3 box is increased roughly 4-fold in the transformed cells relative to the untransformed cell nuclear lysates.

Our data indicate that downregulation of SSeCKS in Src-transformed fibroblasts and in human CaP cells is mediated by the increased binding of Sp1/Sp3 to the GC-box of the SSeCKS promoter which then recruits histone deacetylases (HDAC) such as HDAC1, thereby converting what is an activation complex into a repression complex. Fig. 7 shows Src-transformed cells have relatively increased nuclear levels of Sp1 and Sp3 (~3-4 fold), and Fig. 9 shows that v-Src induces levels of nuclear HDAC1, but not HDAC2 or 3, roughly 3-fold. Indeed, two studies show that HDAC1 levels are increased in human prostate cancer (8;9) although a third study indicates no difference between normal and malignant epithelial cells (10).

In keeping with the increased binding of Sp1 and Sp3 to the GC-box (EMSA assay) as shown in first update, we used chromatin immunoprecipitation (ChIP) analysis to show a similar increase in the association between Sp1/Sp3 and the endogenous SSeCKS promoters (Fig. 10). Interestingly, v-Src induced the enrichment of Sp1, but not Sp3, on the α promoter, whereas it induced the enrichment of Sp3, but not Sp1, on the β promoter. The finding that TSA induces the re-expression of SSeCKS in CaP and Src-transformed cells implies that the suppression of the SSeCKS promoters involves increased recruitment of HDAC isoforms. Indeed, ChIP analysis shows that Src suppresses the association of acetylated histone-3 and –4 (markers of activated or “open” chromatin”) on the α SSeCKS promoter, and that TSA induces their re-association (Fig. 8). The fact that neither Src nor TSA affects the association of Ac-H3 or –H4 with the β SSeCKS promoter suggests some sort of coordinated control directed by the α promoter, as has been suggested for the serum-response elements (5). We have had difficulty in showing increased binding of HDAC1 to the SSeCKS promoters by ChIP assay, likely due to the problems with the existing antibodies, and so we will attempt to show increased HDAC1 binding in the cancer cells by oligonucleotide pulldown using the SSeCKS GC-box domain. We also are attempting to knockdown HDAC1 (or HDAC2 or 3 as controls) by shRNA in order to show that the loss of HDAC1 in the cancer cells results in increased SSeCKS mRNA levels.

We used functional transient expression assays to show that Sp1 and Sp3 alone are inherent activators of the SSeCKS promoter, but when expressed with HDAC1, they become repressors. Specifically, 3T3 or 3T3/v-Src cells transfected with increasing amounts of Sp1 or Sp3 expressor plasmids resulted increased luciferase reporter expression (Fig. 11). Note that this activity in Sp3 required SUMOylation. Fig. 12 shows that transfection of increasing levels of an HDAC1-expressing plasmid alone causes the downregulation of the minimal α SSeCKS promoter activity, but roughly 3-fold more so in Src vs. 3T3 cells. Fig. 13 shows that co-expression of increasing HDAC1 plus either Sp1 or Sp3 resulted in the severe downregulation of the α SSeCKS promoter in both 3T3 and 3T3/v-Src cells. Knockdown of HDAC1 using siRNA (Fig. 14) resulted in increased endogenous transcript levels of both α and β SSeCKS isoforms. We also showed that HDAC1 binds to the αSSeCKS proximal promoter in v-Src-

transformed cells using an oligonucleotide-pulldown assay (Fig. 15). Lastly, using this assay, we pulled down nuclear proteins from P69 and C4-2 cells (Fig. 16), and then subjected the novel bound protein bands to MALDI-TOF analysis. Most interestingly, we identified TFII-I as a novel binding factor to the αSSeCKS proximal promoter. Indeed, TFII-I expression is known to be induced by activated Src (11) and responsible for the downregulation of potential tumor suppressor genes in breast cancer (12).

An interesting finding coming from our now published work on SSeCKS promoter activity in v-Src-transformed cells (13) is that TSA seems to only change the chromatin structure of the α but not the β promoter, yet both promoters are suppressed by v-Src. Moreover, in our hands, the exogenous β promoter could not be downregulated by activated Src in transient expression assays, though the α promoter could. These findings suggest that the β promoter is controlled distally via Src-mediated chromatinization changes >30Kb upstream in the α promoter. This finding seems to be novel in the cancer field in general and suggests that newer generation of HDAC inhibitors in clinical trials (e.g.- SAHA) may exert their anti-cancer effects through the "correction" in gene expression by more long distance changes in chromatin structure.

In sum, we have completed Tasks 1, 3 and 4. We have produced potentially usable indicator CaP cell lines required for Task 2, but induction of significant levels of GFP in these cells by inhibitors of what should be major oncogenic pathways has not been forthcoming. The fact that differentiation agents such as TSA or at-retinoic acid induce GFP expression in these lines leads us to believe we are on the correct track. However, reproducing the reporter construct in DU145 cells failed to show any greater inducibility than in the C4-2 cells. We deem that these cell lines are not suitable for HTS screening given that the maximal induction with relatively broad inhibitors was not more than 3-fold.

Key Research Accomplishments

-construction of SSeCKSα-GFP and TK-RFP reporter plasmids, promoter probes and primer sets to monitor isoform-specific and total SSeCKS transcript levels in human and mouse cells.
-production of indicator CaP and P69 cell lines containing the SSeCKS and TK reporter plasmids.
-development of PCR and Ab-based reagents to detect SSeCKS mRNA and protein expression changes.
-demonstration that SSeCKS/gravin/AKAP12 derepression in CaP cells can be induced by TSA but not by 5-aza-C or 5-deoxy-aza-C.
-demonstration that of the roughly 15-fold decrease in SSeCKS transcript levels in CaP vs. normal cells, 2-fold is controlled by decreases in transcript stability whereas the remaining portion is controlled by a 6- to 8-fold decrease in promoter activity levels.
-demonstration that the minimal CaP- and Src-responsive portion of the SSeCKS promoter is encoded between -106 and +35.
-identification of requirements for both upstream E- and downstream GC-box motifs for downregulation.
-demonstration that the E-box is occupied by USF1 (and not, for example, Myc) and that the GC-box is occupied by a combination of Sp1 and Sp3 (and not, MAZ).

-demonstration that the level of USF1 does not vary in CaP vs. normal cells, but that there is a relative 4-fold increase in Sp3:Sp1 in the transformed cells.
-ChIP assays to show *in vivo* association of SP1, Sp3 and USF with the SSeCKS promoters.
-functional assays showing that the SSeCKS promoters are downregulated by the recruitment of HDAC1 by Sp1/Sp3 in v-Src-transformed cells.
-demonstration that HDAC1 levels are increased in Src-transformed and in CaP cells.
-demonstration that the siRNA knockdown of HDAC1 is sufficient to derepress SSeCKS transcript levels.
-demonstrate increased HDAC1 binding levels on the SSeCKS alpha proximal promoter in cells.
-identify TfII-I by mass spectrometry as an induced protein with enhanced binding activity to the SSeCKS alpha proximal promoter in CaP cells.
-development of first-generation C4-2 and DU145 indicator/reporter cell lines with SSeCKS-GFP and showing inducible expression after TSA or at-retinoic acid treatment.
-publication of data on the Src-responsive SSeCKS promoter sequences and the trans-acting factors involved in SSeCKS downregulation (13) (see Appendix for PDF); preparation of a second manuscript regarding the human promoter sequences and trans-acting factors involved in SSeCKS downregulation in CaP cells.

Reportable Outcomes

-Poster report, 2005 Oncogene Meeting, Frederick, MD, "Mapping of v-Src- and prostate cancer-responsive control sequences to the SSeCKS proximal promoter", Bu, Y. and Gelman, I.H., 6/21-24/2005.
-Poster report, 2006 Annual Meeting, American Assoc. of Cancer Research, Washington, DC, "Identification of v-Src- and prostate cancer-responsive sequences in the promoters of SSeCKS/Gravin/AKAP12, a metastasis-suppressor gene", Bu, Y. and Gelman, I.H., 4/1-5/2006.
-Development of CaP indicator cell lines and probes for SSeCKS isoform expression.

Personnel Involved

-Yahao Bu, Ph.D.- graduate student (completed the work associated with this grant as part of his thesis research, which he defended in July 2007).
-Irwin H Gelman, Ph.D., PI

Conclusions

We have successfully characterized the pathways, mechanisms, promoter sequences and transcription factors involved in the downregulation of SSeCKS promoters in Src-transformed and prostate cancer cells. Novel mechanisms for this downregulation were described, including the possibility that the β promoter is controlled by the cancer-specific regulation of chromatin structure at the α promoter more than Kb upstream. We have also uncovered preliminary data that the recruitment of HDAC1 to a Sp1/Sp3/USF1 complex on the SSeCKS proximal promoter may also recruit TFII-I in order to potentiate the transcriptional suppression of SSeCKS. These data strengthen the notion that the suppression of prostate cancer by treatment with new generation HDAC inhibitors such as SAHA might work through the derepression of SSeCKS. Although we were able to produce C4-2

and DU145 reporter lines based on the SSeCKS promoter, these lines did not show sufficient inducibility of the exogenous promoters to make them useful in HTS protocols, and thus, we did not endeavor to start any drug screens. However, we will continue to attempt to optimize these reporter cells (i.e.- second generation) using data we derived on the molecular characterization of the SSeCKS promoters as well as on the identification of the trans-acting factors involved in transcriptional downregulation in CaP cells.

References

1. **Choi, M. C., H. S. Jong, T. Y. Kim, S. H. Song, D. S. Lee, J. W. Lee, T. Y. Kim, N. K. Kim, and Y. J. Bang**. 2004. AKAP12/Gravin is inactivated by epigenetic mechanism in human gastric carcinoma and shows growth suppressor activity. Oncogene **23**:7095-7103.

2. **Gelman, I. H., E. Tombler, and J. Vargas, Jr.** 2000. A role for SSeCKS, a major protein kinase C substrate with tumor suppressor activity, in cytoskeletal architecture, formation of migratory processes, and cell migration during embryogenesis. Histochemical Journal **32**:13-26.

3. **Lin, X., E. Tombler, P. J. Nelson, M. Ross, and I. H. Gelman**. 1996. A novel *src*- and *ras*-suppressed protein kinase C substrate associated with cytoskeletal architecture. J.Biol.Chem. **271**:28,430-28,438.

4. **Rombouts, K., T. Knittel, L. Machesky, F. Braet, A. Wielant, K. Hellemans, P. De Bleser, I. Gelman, G. Ramadori, and A. Geerts**. 2002. Actin filament formation, reorganization and migration are impaired in hepatic stellate cells under influence of trichostatin A, a histone deacetylase inhibitor. J.Hepatol. **37**:788-796.

5. **Streb, J.W., Kitchen, C.M., Gelman, I.H., Miano, J.M.** 2004. "Multiple promoters direct expression of the three AKAP12 isoforms with distinct subcellular and tissue distribution profiles" J. Biol. Chem. **279**:56014-56023.

6. **Nelson, PS; Clegg, N; Arnold, H; Ferguson, C; Bonham, M; White, J; Hood, L, & Lin, B**. 2002. The program of androgen-responsive genes in neoplastic prostate epithelium Proc. Natl. Acad. Sci. U. S. A, **99**:11890-11895.

7. **Lee, SW; Kim, WJ; Choi, YK; Song, HS; Son, MJ; Gelman, IH; Kim, YJ, & Kim, KW.** 2003. SSeCKS regulates angiogenesis and tight junction formation in blood-brain barrier. Nature Medicine, **9**:900-906.

8. **Halkidou K, Gaughan L, Cook S, Leung HY, Neal DE, Robson CN.** 2004. Upregulation and nuclear recruitment of HDAC1 in hormone refractory prostate cancer. Prostate, **59**:177-89.

9. **Patra SK, Patra A, Dahiya R.** 2001. Histone deacetylase and DNA methyltransferase in human prostate cancer. Biochem Biophys Res Commun, **287**:705-13.

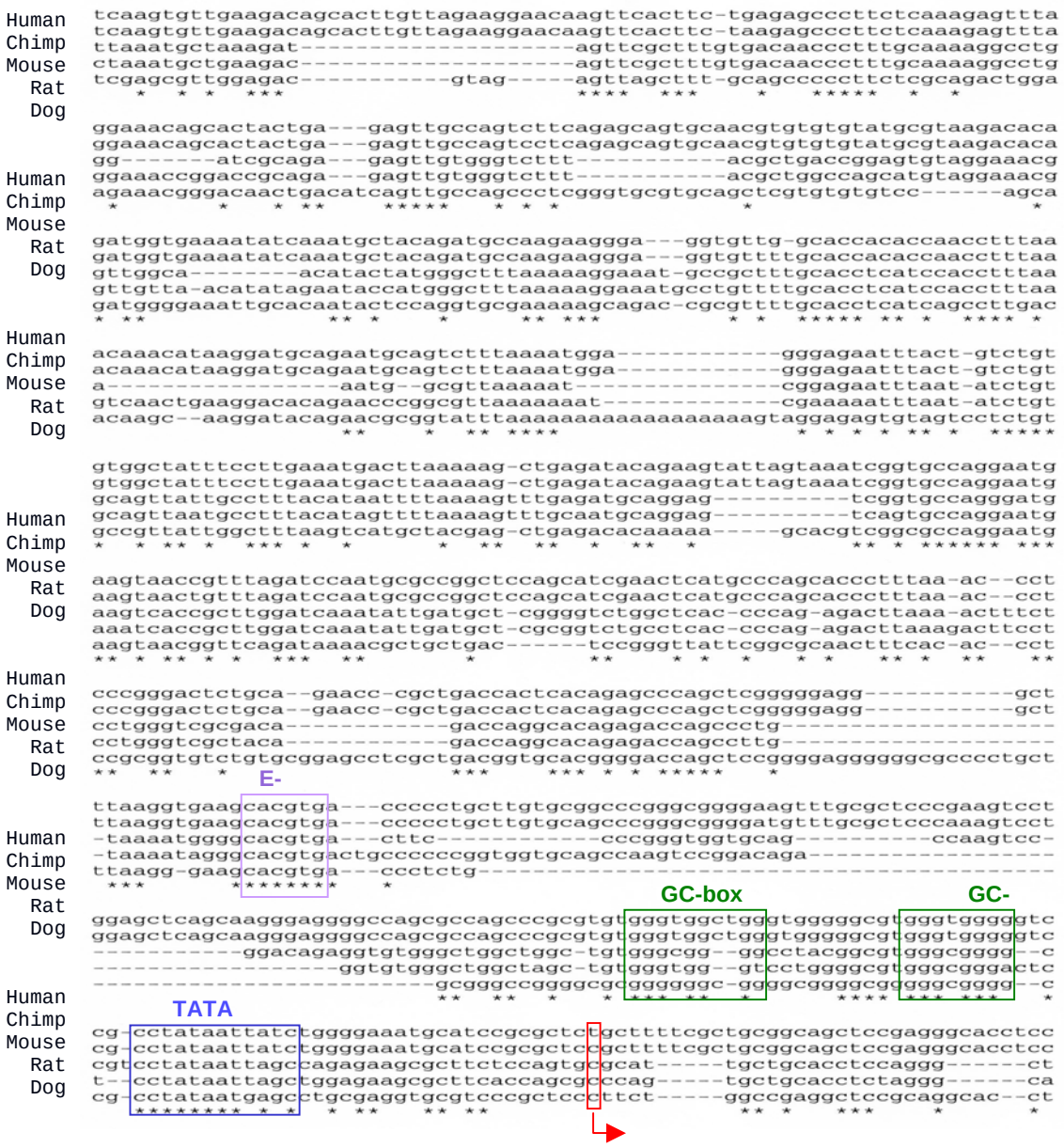
10. **Waltregny D, North B, Van Mellaert F, de Leval J, Verdin E, Castronovo V.** 2004. Screening of histone deacetylases (HDAC) expression in human prostate cancer reveals distinct class I HDAC profiles between epithelial and stromal cells. Eur J Histochem, **48**:273-90.

11. **Cheriyath V, Desgranges ZP, Roy AL.** 2002. c-Src-dependent transcriptional activation of TFII-I. J Biol Chem, **277**:22798-805.

12. **Ogura Y, Azuma M, Tsuboi Y, Kabe Y, Yamaguchi Y, Wada T, et al.** 2006. TFII-I down-regulates a subset of estrogen-responsive genes through its interaction with an initiator element and estrogen receptor alpha. Genes Cells. **11**:373-81.

13. **Bu Y, Gelman IH.** 2007. v-Src-mediated Down-regulation of SSeCKS Metastasis Suppressor Gene Promoter by the Recruitment of HDAC1 into a USF1-Sp1-Sp3 Complex. J Biol Chem. **282**:26725-39.

Appendices



Sequence alignment of SSeCKS α promoter regions in various mammalian species showing strong sequence conservation (*), especially in the retention and spacing of the E- and GC-box motifs just proximal to the transcriptional start site (red).

Supporting Data

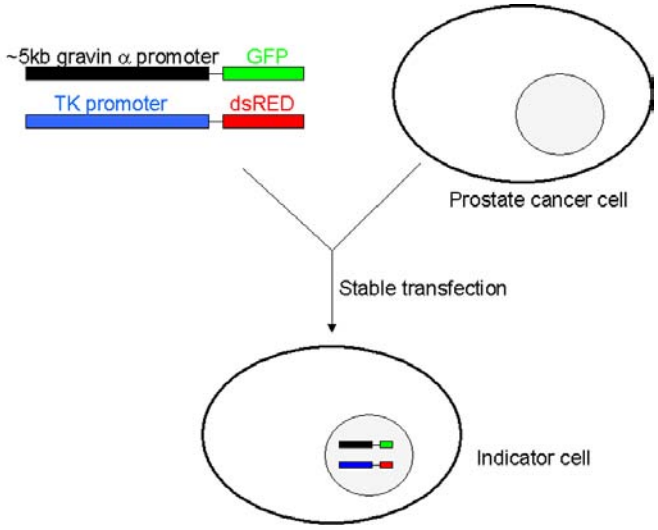


Figure 1. Production of indicator cells lines using the human α SSeCKS/Gravin/AKAP12 promoter.

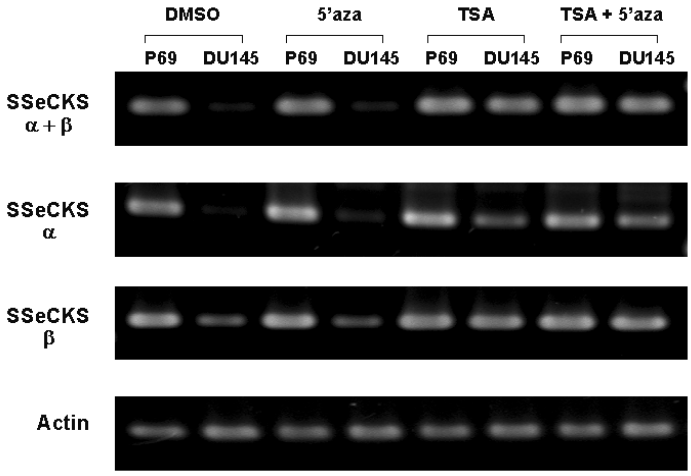


Figure 2. Semi-quantitative RT-PCR of SSeCKS (either the combined α/β, or α or β transcripts, versus actin as a control) showing that TSA, but not 5-azaC, derepresses SSeCKS expression in DU145 cells.

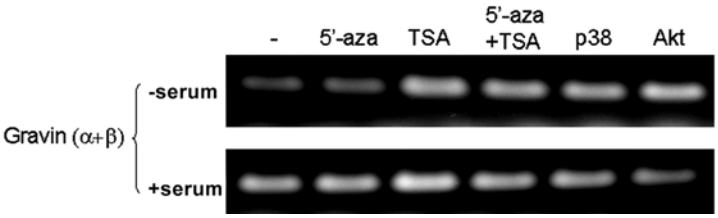


Figure 3. Semi-quantitative RT-PCR of SSeCKS/Gravin α/β from C4-2 cells showing that TSA, the p38 and PI3K inhibitors, but not 5-azaC, derepresses SSeCKS expression only in the absence of serum.

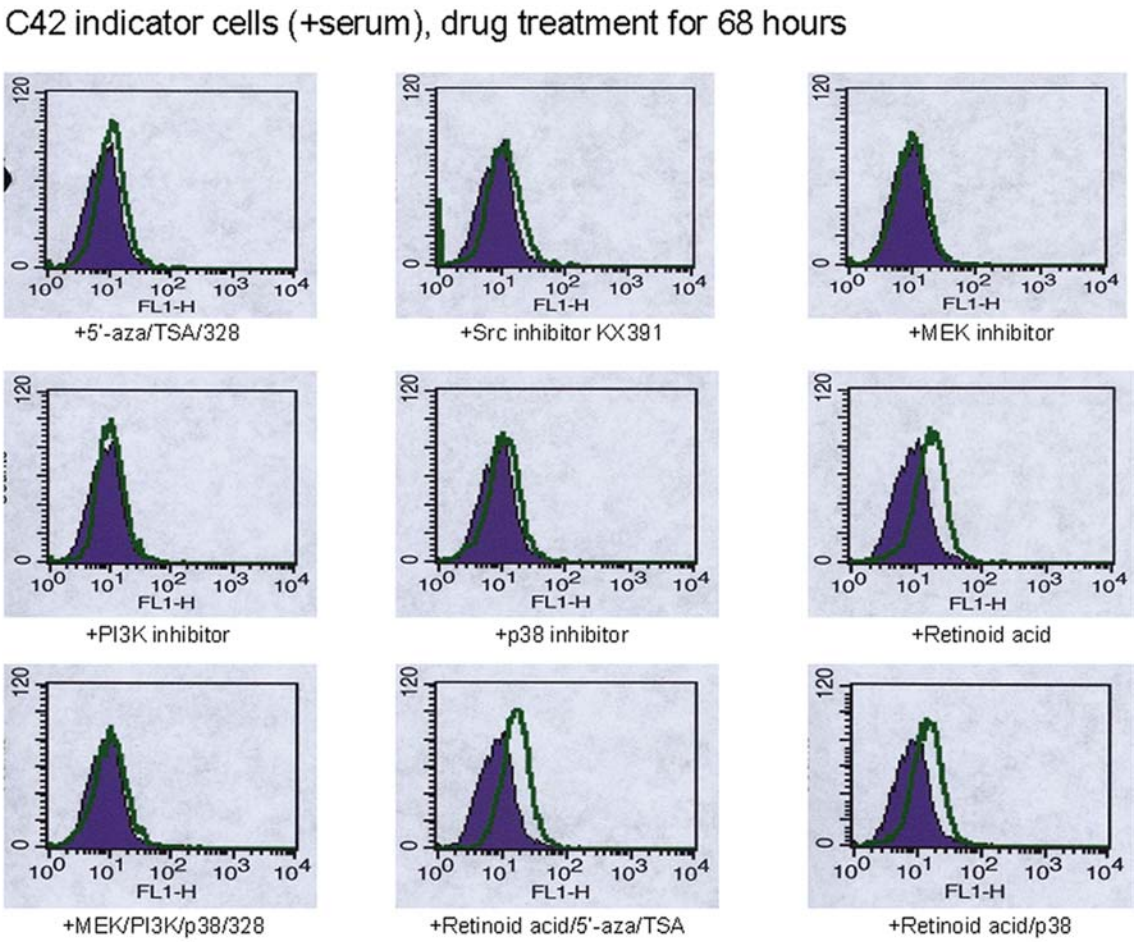


Figure 4. FACS cytometry analysis of GFP expression induced in the C4-2[SSeCKS/GFP] cells line by various signaling inhibitors or differentiation agents.

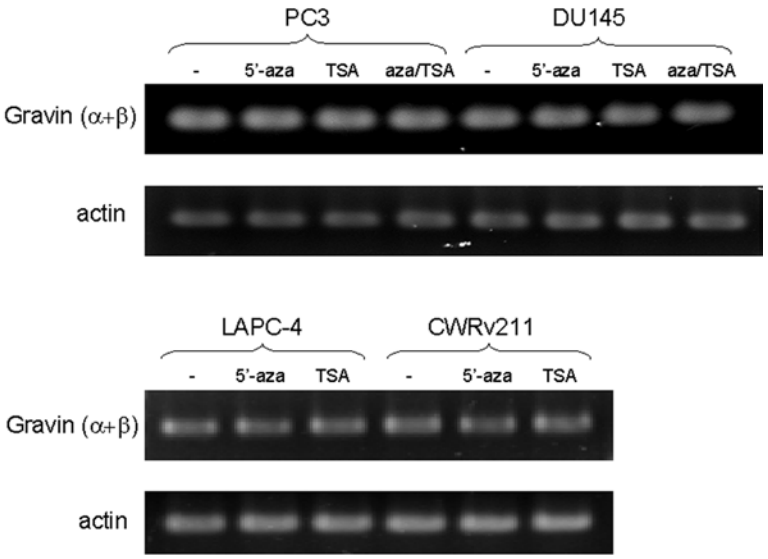
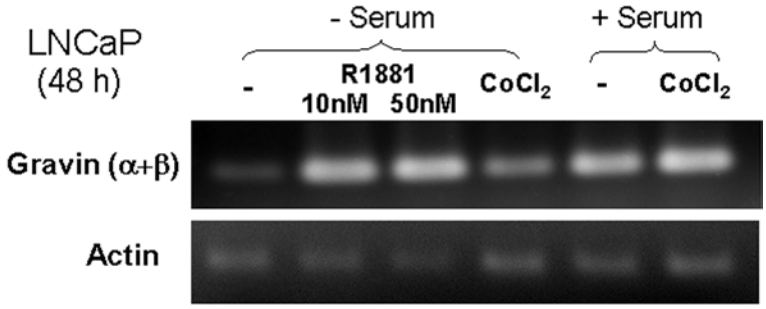


Figure 5. Semi-quantitative RT-PCR of SSeCKS/Gravin α/β (vs. actin control) from CaP cells grown in the presence of serum, showing no SSeCKS derepression by TSA or 5-aza-C.



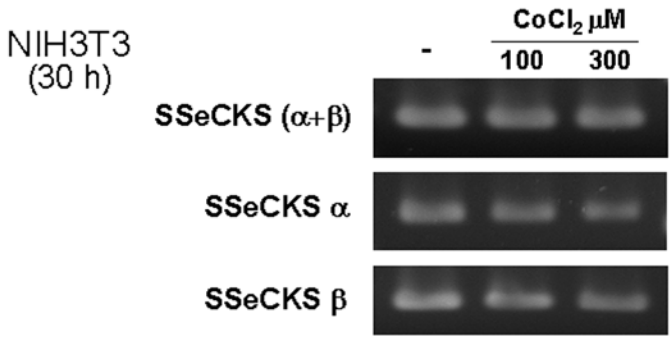


Figure 6. Semi-quantitative RT-PCR of SSeCKS/Gravin α/β (vs. actin control) from LNCaP or NIH3T3 cells treated with either synthetic testosterone (R1881) or $CoCl_2$ (to induce hypoxic conditions).

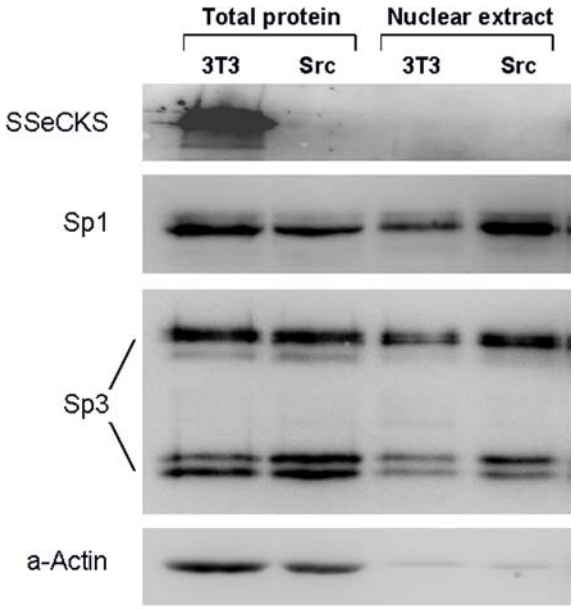


Figure 7. Immunoblotting of 3T3 or 3T3/v-Src nuclear or total cellular lysates for SSeCKS, Sp1, Sp3 and actin. Note the increased relative nuclear levels of Sp1 and Sp3 in Src-transformed cells.

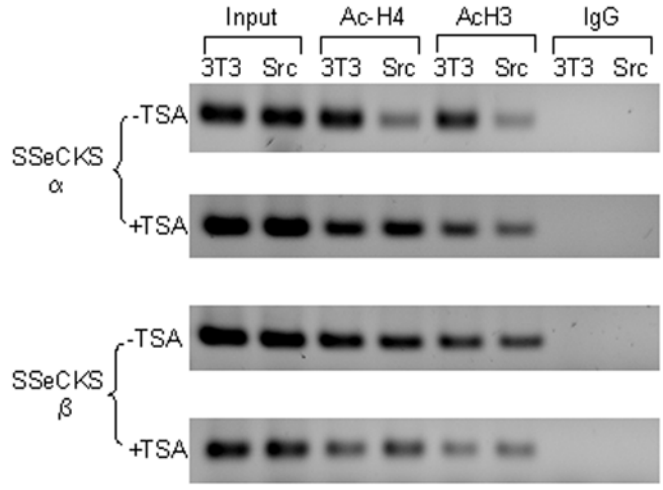


Figure 8. Chromatin immuno-precipitation of 3T3 or 3T3/v-Src nuclear lysates for acetylated histone 3 or 4 (vs. IgG control) followed by PCR amplification for either α or β SSeCKS. Note that Src suppresses the association of the Ac-histones with α but not β SSeCKS, and that TSA induces a re-association, implying that Src induces association of HDAC with the α promoter.

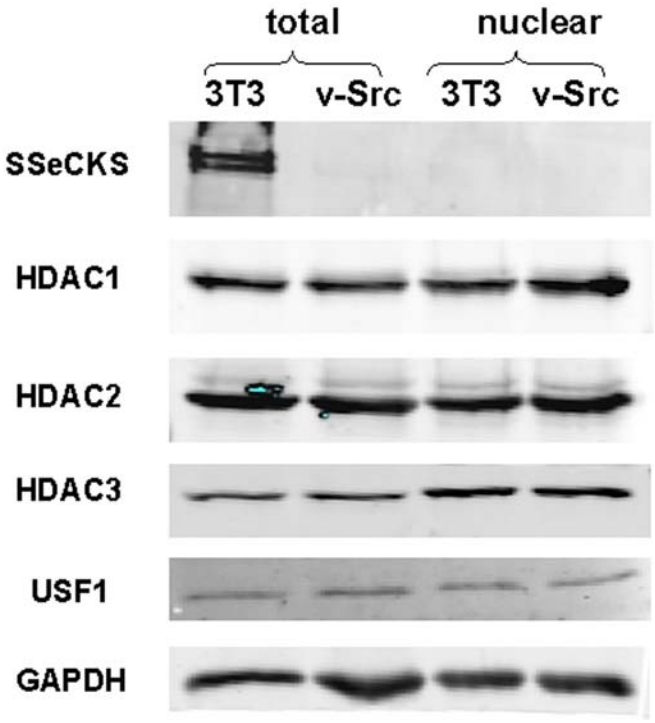


Figure 9. Immunoblotting of 3T3 or 3T3/v-Src nuclear or total cellular lysates for SSeCKS, HDAC1, 2, 3, or GADPH. Note the increased relative nuclear levels HDAC1 in Src-transformed cells.

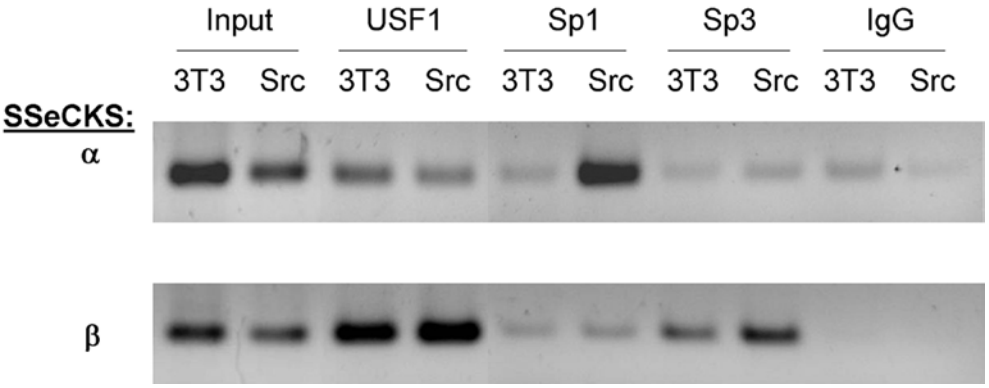


Figure 10. ChIP assay showing increased binding of Sp1 to the α promoter and increased Sp3 binding to the β promoter in 3T3/Src cells.

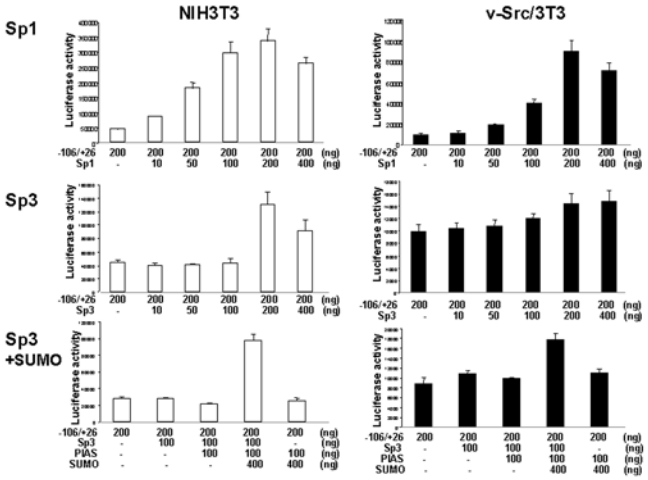


Figure 11. Transient transfection of 3T3 or 3T3/v-Src cells with the minimal αSSeCKS promoter/luciferase along with increasing amounts of Sp1 (top) or Sp3 (middle) expression plasmid, or Sp3 + a SUMO-expression plasmid (bottom).

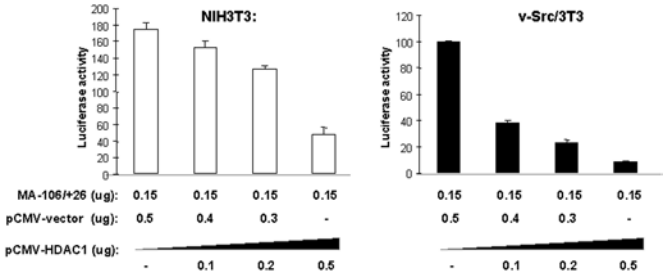


Figure 12. Similar experiment as in Fig. 10 except that the cells were transfected with increasing levels of an HDAC1-expressing plasmid. Note that HDAC1 induces greater relative suppression of the SSeCKS promoter in the Src cells.

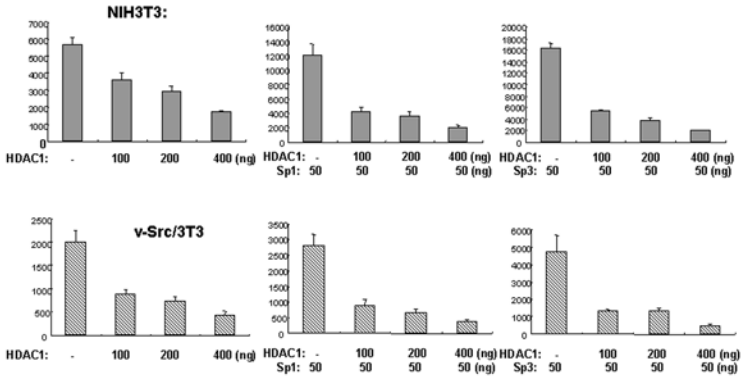


Figure 13. Similar experiment as in Fig. 10 except that the cells were transfected with constant levels of either Sp1- or Sp3-expressing plasmids plus increasing levels of an HDAC1-expressing plasmid.

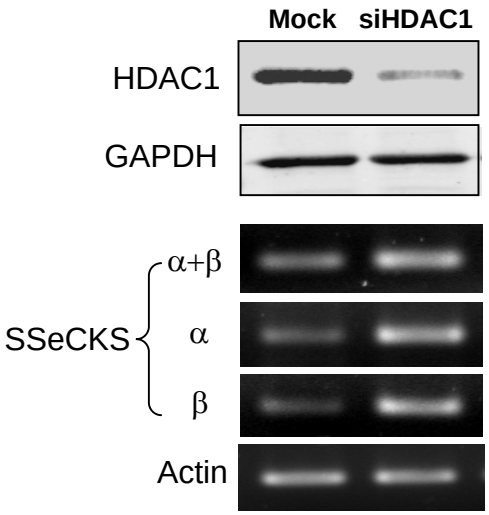


Figure 14. HDAC1 siRNA ("siHDAC1") treatment of NIH3T3/v-Src cells reduces HDAC1 protein levels, compared to GAPDH (top panel), and derepresses endogenous SSeCKS transcript levels.

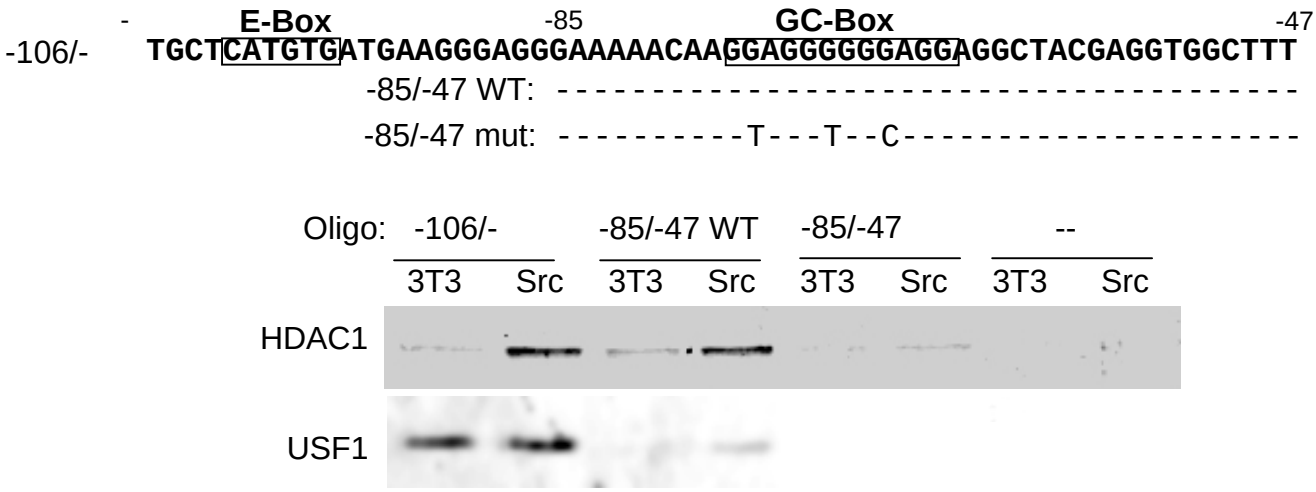


Figure 15. Enrichment of HDAC1 binding to the proximal αSSeCKS promoter. Nuclear lysates from NIH3T3 and NIH3T3/v-Src cells were incubated with double-stranded biotin-labeled oligonucleotides representing the WT or mutated ("mut") αSSeCKS promoter region from –85 to –47. The DNA/protein complexes were then bound to streptavidin beads, washed and then probed by IB for HDAC1 or USF1.

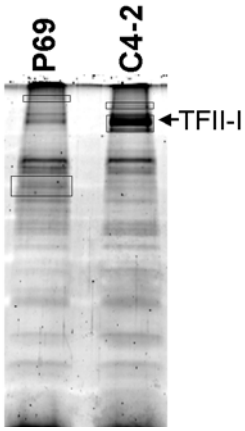


Figure 16. Binding of TFII-I to the proximal αSSeCKS promoter in CaP. Using the oligonucleotide-pulldown assay described in Fig. 15 (with WT –85/-47), proteins that bind to the proximal αSSeCKS promoter were isolated from nuclear lysates from P69 and C4-2 cells. The bands shown as rectangles were excised after staining of the proteins in the gel and then subjected to MALDI-TOF after complete trypsin digestion. This analysis identified the band in C4-2 cells as being TFII-I.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 37, pp. 26725–26739, September 14, 2007

v-*Src*-mediated Down-regulation of SSeCKS Metastasis Suppressor Gene Promoter by the Recruitment of HDAC1 into a USF1-Sp1-Sp3 Complex*[S]

Received for publication, April 4, 2007, and in revised form, June 6, 2007 Published, JBC Papers in Press, July 10, 2007, DOI 10.1074/jbc.M702885200

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SSeCKS (Src-suppressed C kinase substrate), also called gravin/AKAP12, is a large scaffolding protein with metastasis suppressor activity. Two major isoforms of SSeCKS are expressed in most cell and tissue types under the control of two independent promoters, designated α and β, separated by 68 kb. SSeCKS transcript and protein levels are severely decreased in Src- and Ras-transformed fibroblasts and in many epithelial tumors. By dissecting its promoters with progressive deletion analysis, we identified the sequence between −106 and −49 in the α proximal promoter as the minimal v-*Src*-responsive element, which contains E- and GC-boxes bound by USF1 and Sp1/Sp3, respectively. Both E- and GC-boxes are crucial for v-*Src*-responsive and basal promoter activities. v-*Src* does not alter USF1 binding levels at the E-box, but it increases Sp1/Sp3 binding to the GC-box despite no change in their cellular protein abundance. SSeCKS α and β transcript levels in v-*Src*/3T3 cells can be restored by treatment with the histone deacetylase inhibitor, trichostatin A, but not with the DNA demethylation agent, 5-azacytidine. Chromatin changes are found only on the α promoter even though the β proximal promoter contains a similar E- and GC-box arrangement. Recruitment of HDAC1 is necessary and sufficient to cause repression of α proximal promoter activity, and the addition of Sp1 and/or Sp3 potentiates the repression. Our data suggest that suppression of the β promoter is facilitated by Src-induced changes in the α promoter chromatinization mediated by a USF1-Sp1-Sp3 complex.

SSeCKS (Src-suppressed C kinase substrate) was originally identified in a screen for genes severely down-regulated by v-*Src* (1), and then characterized as a major *in vitro* and *in vivo* substrate of protein kinase C (2). SSeCKS is the rodent orthologue of human gravin, a kinase scaffold protein originally discovered as an autoantigen in some myasthenia gravis patients (3, 4). Based on their ability to bind protein kinase A RII isoforms (3), SSeCKS and gravin have been re-designated AKAP12 (A kinase anchoring protein 12). In addition to protein kinase A and protein kinase C, SSeCKS also binds with calmodulin, cyclin D1, β-adrenergic receptor, β-1,4 galactosyltransferase, and F-actin (5). With its ability to scaffold key signaling and cytoskeletal proteins, SSeCKS plays important roles in regulating $G_1 \rightarrow S$ phase transition and cytoskeletal organization (6, 7). Additionally, several lines of evidence suggest that SSeCKS/gravin/AKAP12 (heretofore called "SSeCKS") functions as a suppressor of tumorigenesis and metastasis. The expression of SSeCKS is down-regulated by several oncogenes and in various human epithelial tumors, including prostate, breast, ovarian, gastric, and lung (8–13). The locus of human *GRAVIN* gene in chromosome 6q24–25.2 is a deletion hotspot in advanced prostate, breast, and ovarian cancers (8, 14). Moreover, SSeCKS re-expression in Src-transformed fibroblasts can reverse Src-induced oncogenic growth by reducing anchorage-independent proliferation and inhibiting metastatic invasiveness through the suppression of podosome formation, most likely via the reestablishment of normal cytoskeletal architecture and the suppression of Rho family GTPase activity (15, 16). The re-expression of SSeCKS in rat Mat-LyLu prostate cancer cells slightly reduces the growth rate of primary subcutaneous tumors in nude mice but greatly suppresses the formation of lung metastases by decreasing angiogenesis through the inhibition of expression of pro-angiogenic factors such as vascular endothelial growth factor (17). The *SSeCKS* gene locus in human and rodents encodes three major transcripts under the control of three independent promoters, designated α, β, and γ, that are separated by 84 kb (5, 18). Two major protein isoforms of SSeCKS, α and β, are expressed ubiquitously in most cell and tissue types (5), whereas the expression of isoform γ is testes-restricted (19, 20). All proteins encoded by each transcript share >95% amino acid sequence identity encoded by a single large exon but differ only at their extreme N-terminal residues. For example, only the α isoform encodes a product that is myristoylated (2), and this modification facilitates α SSeCKS association with plasma membranes and vesicles of the endoplasmic reticulum (7, 18, 21), yet it is not sufficient for its plasma membrane targeting (22).

The mechanism by which SSeCKS is down-regulated in tumor cells has not been studied. The fact that SSeCKS is down-regulated by some oncogenes (*Src, Ras, Myc,* and *Jun*) but not by others (*Raf*, *Mos*, or *Neu*) suggests that this is not a generic effect in transformed cells but rather is controlled by specific mitogenic and oncogenic pathways (5). Given that down-regulation of SSeCKS is not a bystander effect during oncogenic transformation, understanding the molecular mechanism

* This work was supported by National Institutes of Health Grant CA94108 and Department of Defense Grant PC040256 (to I. H. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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involved in SSeCKS gene silencing in the course of tumorigenesis will contribute to control tumor progression by means to reactivate SSeCKS expression.

In this study, we dissect the *cis*- and *trans*-factors responsible for the v-*Src*-induced repression of *ssecks* promoters. We find that the minimal v-*Src*-responsive element (VSRE)[2] requires both E- and GC-boxes in the SSeCKS α proximal promoter (-106 to -49), which are bound by the transcription factors USF1 and Sp1/3, respectively. Our data indicate that v-*Src*-mediated transcriptional repression correlates with increased complex formation between USF1 and Sp1/3, increased binding activity of Sp1/3 to the α SSeCKS VSRE, and the recruitment of HDAC1. Moreover, although the mouse and human α and β promoters share E- and GC-boxes in their proximal promoter, our data suggest that suppression of the β promoter is facilitated by chromatin structure change in the α promoter 68 kb upstream.

EXPERIMENTAL PROCEDURES

Cell Culture—NIH3T3 mouse fibroblasts were maintained in Dulbecco's modified Eagle's medium containing 10% bovine serum. v-*Src*-transformed NIH3T3 cells (v-*Src*/3T3) were generated by retrovirus-mediated transduction. Briefly, pBabe-v-*Src*/puro DNA was transfected into the 293GPG packaging cell line (23) using Lipofectamine 2000 (Invitrogen). Virus was harvested 72 h after transfection and used to infect NIH3T3 cells (10^4/well in 12-well dishes) for 4 h. Cells were maintained in selection medium containing 2 μg/ml puromycin (Sigma). Single colonies with transformed cell morphology were picked and expanded, and then the expression of oncogenic v-*Src* was verified by Western blot with anti-v-Src (avian) mAb EC10, anti-Src[poY^{416}] (BIOSOURCE), and anti-phosphotyrosine mAb-4G10 (Upstate, Charlottesville, VA) antibodies. v-*Src*/3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% bovine serum and 2 μg/ml puromycin.

Immunofluorescence Staining—NIH3T3 or v-*Src*/3T3 cells grown to 60% confluence on 22-mm coverslips were fixed with 4% paraformaldehyde in PBS for 10 min and permeabilized with 0.1% Triton X-100 in PBS for another 10 min. After rinsing with PBS, cells were stained with rhodamine-conjugated phalloidin and 4′,6-diamidino-2-phenylindole (1:500 dilution) (Invitrogen) for 1 h at room temperature. After three PBS washes, coverslips were mounted on glass slides in ProLong antifade reagent (Invitrogen). Fluorescent images were captured using a Nikon TE2000-E inverted microscope (Garden City, NY).

Primer Extension Analysis—Total RNA from NIH3T3 cells was extracted using TRIzol (Invitrogen) following the manufacturer's instructions. A ^{32}P-end-labeled antisense primer (for SSeCKS α, 5′-AGGAGATGTGCGCCCAGGACCACAGG-3′; for SSeCKS β, 5′-CCTTCTCCTCTGTCTACTCCCGGCTA-ACC-3′) corresponding to $+66/+91$ or $+72/+101$ relative to the transcriptional start site of SSeCKS α or β, respectively, was hybridized at 58 °C overnight with 50 μg of total RNA (previously denatured for 3 min at 90 °C). The annealed primer was extended with SuperScript II reverse transcriptase (Invitrogen) at 42 °C for 1 h. The sizes of the extension products were determined by electrophoresis on 8% denaturing polyacrylamide gel containing 7 M urea. A 10-bp ^{32}P-labeled ladder and sequencing reaction performed with the same primer on a genomic clone was used as a reference. The gel was dried, and the radioactive signals were identified by phosphorimaging (Storm-860, GE Healthcare).

Reporter Constructs—The SSeCKS α promoter sequence ($-4920/+36$) and β promoter sequence ($-4758/+119$) were amplified from the BAC clone (RP11-27244) using the TripleMaster long run PCR system (Eppendorf, Westbury, NY) and then ligated into pCR-XL-TOPO vector (Invitrogen). The MluI/XhoI fragments from these plasmids were subcloned into the luciferase reporter vector pGL3-Basic (Promega, Madison WI) cut with MluI/XhoI. Progressive deletion mutants of SSeCKS promoter-luciferase constructs were created by inverse PCR with promoter-specific, MluI-flanked primers (Table 1), using the ~5-kb SSeCKS promoter/luciferase constructs as templates, followed by self-ligation. All constructs were validated by DNA sequencing.

Promoter/reporter constructs with mutations in the E-box and/or GC-box were generated using the QuikChange site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) according to manufacturer's instructions, using the $-106/+36$ α SSeCKS reporter construct as template. Nucleotide changes are indicated in italics for each primer (Table 1). All reporter mutations were confirmed by DNA sequencing.

For the SSeCKS-TK heterologous promoter constructs, a minimal TK promoter sequence was amplified from pRL-TK (Promega) with XhoI- or HindIII-flanked primers (Table 1). The PCR products were digested with XhoI/HindIII and cloned into pGL3-Basic vector cut with XhoI/HindIII to create the TKm-pGL3B luciferase reporter plasmid. The wild type SSeCKS α proximal promoter sequence between -106 and -49 and proximal promoters containing mutated E-Box and/or GC-Box were amplified with the KpnI- or XhoI-flanked primers (Table 1), digested with KpnI and XhoI, and inserted into TKm-pGL3B plasmid cut with KpnI/XhoI.

Reverse Transcription (RT)-PCR—Total RNA was isolated from NIH3T3 and v-*Src*/3T3 cells with or without treatment of fresh 5-azacytidine (5-aza-C) (500 nM for 72 h) and/or trichostatin A (TSA) (330 nM for 24 h) (Sigma) using TRIzol (Invitrogen). 1 μg of total RNA was subjected to reverse transcription using SuperScript first-strand synthesis system kit (Invitrogen) according to manufacturer's instructions. PCR was then performed using MJ Research PTC-200 DNA thermal cycler (Watertown, MA) with the optimized cycle numbers for each primer set (Table 1). The PCR products were separated by electrophoresis through a 1.5% agarose gel and digitally imaged using a Chemi-Genius[2] Bio-Imager (Syngene, Frederick, MD).

[2] The abbreviations used are: VSRE, v-*Src*-responsive element; 5-aza-C, 5-azacytidine; Ac-H3/H4, acetylated histone H3/H4; AKAP, A kinase anchoring protein; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; HDAC, histone deacetylase; TSA, trichostatin A; TSS, transcription start site; VSR, v-*Src*-responsive; Ab, antibody; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; HA, hemagglutinin; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, small interfering RNA; WT, wild type; TK, thymidine kinase.

mRNA Stability Analysis—NIH3T3 and v-*Src*/3T3 cells (2×10^5) seeded in 35-cm dishes were harvested at 2, 4, 8, 16, and 24 h after addition of actinomycin D (5 μg/ml) (Sigma). Total cellular RNA was extracted, and RT-PCR was performed as described above. Because the endogenous SSeCKS mRNA levels are suppressed in v-*Src*/3T3 cells, the PCR cycle numbers for SSeCKS α were 30 for NIH3T3 cells and 38 for v-*Src*/3T3 cells; for SSeCKS β were 29 for NIH3T3 cells and 34 for v-*Src*/3T3 cells; and for the shared α/β sequence were 28 for NIH3T3 cells and 32 for v-*Src*/3T3 cells.

Western Blot Analysis—Whole cell lysates were prepared in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate) with freshly added inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM NaF, 1 mM Na_3VO_4) and protease inhibitor mixture (Roche Applied Science). The protein concentration was determined by the Bradford protein assay (Bio-Rad). Proteins were separated on 10% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (PerkinElmer Life Sciences). The membranes were blocked with 5% nonfat milk in TBST (150 mM NaCl, 100 mM Tris-HCl, pH 7.4, 0.1% Tween 20), or with 5% bovine serum albumin in TBST for phosphoprotein blots. The following primary antibodies were used as indicated: anti-SSeCKS (2), anti-v-Src mAb EC10 (gift of Sarah Parsons, University of Virginia), anti-Src[poY^{416}] (BIOSOURCE), anti-phosphotyrosine mAb-4G10 (Upstate), anti-FLAG and anti-actin clone AC40 (Sigma), anti-GAPDH, anti-lamin A/C, anti-USF1, anti-Sp1, anti-Sp3, anti-HDAC1, anti-HDAC2, anti-HDAC3, anti-PIAS1, and anti-HA tag (Santa Cruz Biotechnology, Santa Cruz, CA). After washing and incubating with horseradish peroxidase-labeled anti-rabbit or -mouse IgG secondary antibodies, the blots were washed, incubated with Lumi-Light chemiluminescence reagent (Roche Applied Science), and digitally imaged using a Chemi-Genius^2 Bio-Imager.

Transient Transfection and Luciferase Assay—All transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For reporter assays, cells were seeded in 24-well plates at a density of 3×10^4 cells/well 24 h prior to transfection. Each transfection was performed in triplicate with 0.2 μg of promoter/reporter constructs along with 0.1 μg of *Renilla* luciferase reporter pRL-TK (Promega), used to normalize for transfection efficiency. Cells were harvested 48 h after transfection and lysed, and luciferase activities were measured using the dual luciferase assay kits (Promega). For overexpression experiments, a set amount of the SSeCKS α proximal promoter-reporter construct ($-106/+36$) was transfected in combination with various expression plasmids as indicated. The total amount of DNA transfected was normalized using the appropriate empty vectors. Data presented are representative of at least three independent experiments. The Sp1 expression vector (pFLAG-Sp1-HA) was a generous gift from Dr. Adrian Black (Roswell Park Cancer Institute). Expression vectors of pCMV-Sp3 and pN3-PIAS1, encoding a SUMO ligase, were kind gifts from Professor Guntram Suske (Institute für Molekularbiologie and Tumorforschung, Marburg, Germany). The expression vector for HA tagged SUMO-1 cDNA (HA-Sen1) was a gift kindly provided by Dr. Edward T. H. Yeh (University of Texas M. D. Anderson Cancer Center, Houston, TX). The HA-tagged HDAC1 expression plasmid (pCMV-2N3T-HDAC1) was a generous gift from Dr. Didier Trouche (Université Paul Sabatier, France).

Preparation of Nuclear Extracts—Nuclear extracts were prepared essentially as described previously (24) with minor modifications. Briefly, cells growing in 10-cm dishes (80–90% confluence) were washed twice with cold PBS and scraped into 1.5 ml of PBS. Cells were collected by centrifuging at 2,000 rpm for 2 min and then resuspending in 1 ml of ice-cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.1% Nonidet P-40, 1 mM PMSF, and Roche Applied Science protease inhibitor mixture). After incubating on ice for 20 min, the cells were homogenized with a glass Dounce (type A) by applying 30 strokes. Nuclei were collected by centrifugation at 4,000 rpm for 15 min at 4 °C. The pellets were resuspended in 200 μl of buffer C (20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 1 mM DTT, 1 mM PMSF, and Roche Applied Science protease inhibitor mixture) and incubated on ice for 30 min with occasional agitation. The nuclear extract was centrifuged at 13,000 rpm for 20 min at 4 °C, and the supernatant was stored in aliquots at −80 °C. Protein concentrations of nuclear extracts were determined using the Bradford protein assay (Bio-Rad).

Electrophoretic Mobility Shift Assay (EMSA)—All DNA oligonucleotides used for EMSA were synthesized by Integrated DNA Technologies (Coralville, IA). Oligonucleotides were annealed, end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase, and purified by passage through Sephadex G-50 microcolumns (Amersham Biosciences). For each reaction, 5 μg of nuclear extract was preincubated with 1.5 μg of poly(dI-dC) (Sigma) for 20 min on ice in 10 μl of binding buffer (10 mM HEPES, pH 7.9, 5 mM KCl, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 10% glycerol). 0.2 ng of ^{32}P-labeled double-stranded oligonucleotides (~20,000 cpm) was added to the 10-μl reaction mixtures and incubated for 30 min at room temperature, and then electrophoresed on 4% nondenaturing polyacrylamide gels in 0.5× Tris borate/EDTA buffer run at 4 °C. The gels were sandwiched with Whatman 3M paper, dried, and then autoradiographed overnight with an intensifying screen. In competition or supershift assays, molar excess amounts of unlabeled DNA probe or 2 μg of antibody were added to the preincubation mixtures 20 min or 1 h, respectively, prior to the addition of ^{32}P-labeled DNA oligonucleotides.

DNA Affinity Precipitation Assay—Nuclear extracts (100 μg) from NIH3T3 or v-*Src*/3T3 cells were incubated at room temperature for 15 min with 0.5 nmol of 5′-biotinylated double-stranded oligonucleotides (Integrated DNA Technologies), which were previously conjugated to streptavidin-agarose beads (Sigma), in 300 μl of low salt lysis buffer (1% Triton X-100, 0.1% sodium deoxycholate, 0.05 M Tris-HCl, pH 8.1, 5 mM EDTA, pH 8.0) containing 1 mM PMSF and Roche Applied Science protease inhibitor mixture. The beads were then washed six times with low salt lysis buffer containing 150 mM NaCl and boiled in 1× electrophoresis sample buffer to elute the bound proteins before running on an SDS-polyacrylamide gel. The separated proteins were transferred to polyvinylidene

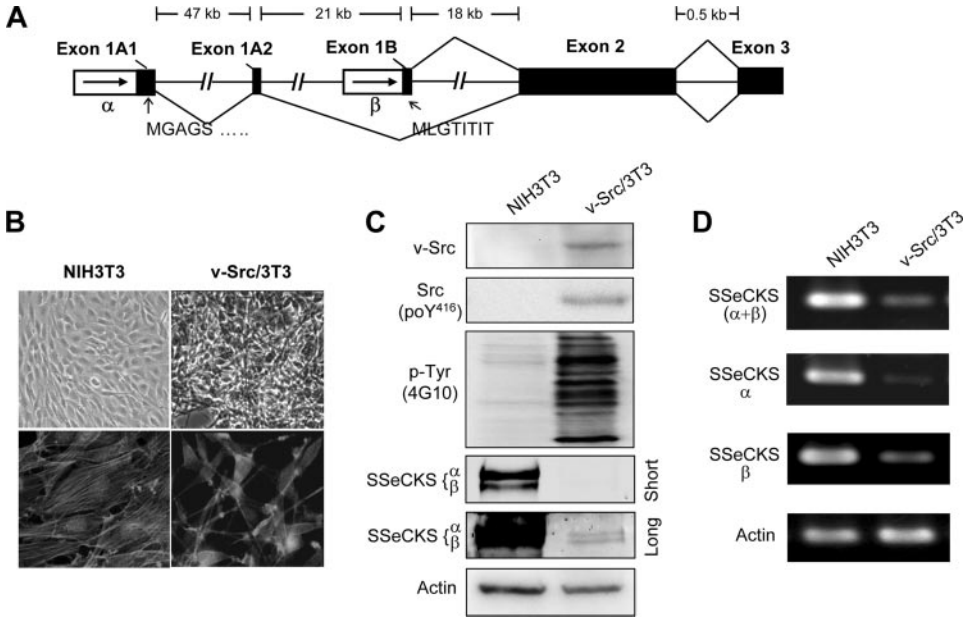


FIGURE 1. **SSeCKS transcript and protein levels are severely down-regulated by v-*Src*.** *A*, schematic diagram of mouse SSeCKS gene structure. Exons 1A1 and 1A2 encode a 103-amino acid myristoylated N-terminal domain of the α SSeCKS isoform driven by the α promoter, whereas exon1B encodes an 8-amino acid nonmyristoylated domain of the β isoform driven by the β promoter. Both are fused to a common exon2 sequence encoding roughly 1500 additional amino acids. *B*, phase-contrast images of NIH3T3 and v-*Src*/3T3 cells at confluence (*upper panel*) and immunofluorescence staining of F-actin with rhodamine-conjugated phalloidin and of nuclei with 4′,6-diamidino-2-phenylindole (*lower panel*). *C*, cell lysates prepared from NIH3T3 and v-*Src*/3T3 cells were immunoblotted with antibodies specific for avian Src (mAb-EC10), Src^{poY416}, total phosphotyrosine, SSeCKS, or actin (as a loading control). Both *short* and *long* exposures are presented to show that both SSeCKS isoforms are present in v-*Src*/3T3 lysates. *D*, steady-state SSeCKS mRNA levels in NIH3T3 and v-*Src*/3T3 cells were determined by semi-quantitative RT-PCR using isoform-specific or common α/β primers. Actin-specific primers were used as a control.

difluoride membrane and immunoblotted with antibodies against HDAC1 and USF1 (Santa Cruz Biotechnology).

Chromatin Immunoprecipitation (ChIP)—ChIP assays were performed following the protocol outlined by the manufacturer (Upstate) with minor modifications. Briefly, NIH3T3 and v-*Src*/3T3 cells growing in 10-cm dishes (80–90% confluence) were fixed in culture medium with formaldehyde (final concentration of 1%) for 10 min at 37 °C. After washing twice with cold PBS, cells were collected by centrifuging at 2,000 rpm for 2 min, resuspended in 0.8 ml of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) containing 1 mM PMSF and Roche Applied Science protease inhibitor mixture, and incubated for 20 min on ice. DNA was sheared into ~200–1000-bp fragments by five 20-s sonications, followed by centrifugation for 10 min at 13,000 rpm at 4 °C to remove debris. Supernatant fractions were diluted 5-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl) containing 1 mM PMSF and Roche Applied Science protease inhibitor mixture. Each chromatin fraction (1.2 ml per immunoprecipitation) was precleared with 60 μl of salmon sperm DNA-protein A-agarose beads (Upstate) for 1 h at 4 °C and then incubated at 4 °C overnight with 5 μg of the following antibodies as indicated: anti-USF1, anti-Sp1, anti-Sp3, and normal rabbit IgG (Santa Cruz Biotechnology), anti-acetyl-histone H4 and anti-acetyl-histone H3 (Upstate). Immune complexes were isolated by binding to 40 μl of salmon sperm DNA-protein A-agarose beads for 1 h at 4 °C, and by washing sequentially with low salt buffer (50 mM Tris-HCl, pH 8.0, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 1 mM EDTA, 150 mM NaCl), high salt buffer (50 mM Tris-HCl, pH 8.0, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 1 mM EDTA, 500 mM NaCl), LiCl buffer (50 mM Tris-HCl, pH 8.0, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 1 mM EDTA, 250 mM LiCl), and then twice with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). DNA-protein complexes were eluted twice with 250 μl of 1% SDS and 0.1 M $NaHCO_3$ and incubated at 65 °C for 4 h to reverse the cross-linking. Proteins were digested with proteinase K, and DNA was recovered by phenol/chloroform extraction and ethanol precipitation with 20 μg of glycogen as carrier. Primers for PCR amplification of SSeCKS α proximal promoter sequence between −270 and +33 were 5′-TGCTGCTCCTGAACCTTCTG-3′ and 5′-GATCCTGCTGAGAACACACC-3′. SSeCKS β proximal promoter sequence between −248 and +43 were 5′-GTGCCAGGGATGAAGTCACC-3′ and 5′-GAGCATCAAGGAAGCTCTCC-3′. PCR products were resolved on 2% agarose gels and stained with ethidium bromide, and the images were digitized with a Chemi-Genius^2 Bio-Imager.

siRNA Experiments—ON-TARGET plus SMART pool siRNA specific for murine HDAC1 was purchased from Dharmacon (Lafayette, CO). v-*Src*/3T3 cells plated in 6-well plates (1×10^5 cells/well) were transfected with 200 nM siRNA-HDAC1 using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. After 72 h, cells were harvested for Western blot analysis and semi-quantitative RT-PCR.

RESULTS

SSeCKS mRNA and Protein Levels Are Severely Down-regulated in v-Src-transformed NIH3T3 Cells—The mouse *ssecks* gene locus encodes two major transcript isoforms, α and β (the testes-specific isoform γ was not studied here), under the control of two independent promoters. As shown in Fig. 1*A*, exon 1A1 and exon 1A2 encode a 103-amino acid myristoylated N-terminal domain driven by the TATA-less α promoter. Exon1B encodes an 8-amino acid nonmyristoylated domain driven by the TATA-containing β promoter. Both are fused to the common exon 2 encoding the remaining 1494 amino acids, and exon 3, which contains the 3′-untranslated region. The α and β promoters are separated by 68 kb. Compared with untransformed NIH3T3 cells, NIH3T3 cells transduced with the v-*Src* oncogene (v-*Src*/3T3) are refractile, deficient in contact-inhibited growth, and lack F-actin stress fibers (Fig. 1*B*).

TABLE 1
PCR primers

	Forward primer	Reverse primer
SSeCKS α promoter		
−4920/+36	GACAAGTTCAGCCTGCTCTTCC	CACGATCCTGCTGAGAACAC
−3239/+36	CCGACGCGTATGCATTAGAAGAGGGCGT	TGCATAGCTTGAGTATTCTAACGC
−2677/+36	CCGACGCGTTTCGAGACAGGGTTTCTCTG	TGCATAGCTTGAGTATTCTAACGC
−1455/+36	CCGACGCGCGTGCTAGCCTGAACTAGATTGTG	TGCATAGCTTGAGTATTCTAACGC
−636/+36	CCGACGCGTCCCGGGGAAAGAGGGCGGCG	TGCATAGCTTGAGTATTCTAACGC
−270/+36	CCGACGCGTTGCTGCTCCTGAACCTTCTG	TGCATAGCTTGAGTATTCTAACGC
−106/+36	CCGACGCGTTGCTCATGTGATGAAGGGAG	TGCATAGCTTGAGTATTCTAACGC
−89/+36	CCGACGCGTGAGGGAAAACAAGGAGGG	TGCATAGCTTGAGTATTCTAACGC
−67/+36	CCGACGCGTAGAGGCTACGAGGTGGCTT	TGCATAGCTTGAGTATTCTAACGC
−43/+36	CCGACGCGTTTTTTTTTTTCTTTTAAGG	TGCATAGCTTGAGTATTCTAACGC
−26/+36	CCGACGCGTAGGAGTTGCCACGTAGCG	TGCATAGCTTGAGTATTCTAACGC
SSeCKS β promoter		
−4758/+119	TGTGCACATTACGCATGGAGG	CTCTTACGGCAGGAAGTCTCC
−2482/+119	CCGACGCGTTGGGAGAGACTCATGTAGCC	TGCATAGCTTGAGTATTCTAACGC
−551/+119	CCGACGCGTCCACGAAAAGTAGAGAGACC	TGCATAGCTTGAGTATTCTAACGC
−157/+119	CCGACGCGTAGACCAGGCACAGAGACCAG	TGCATAGCTTGAGTATTCTAACGC
−24+119	CCGACGCGTAGCCAGAGAAGCGCTTCTCC	TGCATAGCTTGAGTATTCTAACGC
+16/+119	CCGACGCGTCCAGGGCTGGAGAGCTTCCTTG	TGCATAGCTTGAGTATTCTAACGC
SSeCKS α −106/+36 mutagenesis[a]		
MutE-box	CTTACGCCGCGTTGT*T*CA*AA*TGATGAAGG	CCTTCATCA*TT*TGAACAACGCGGCGTAAG
MutGC-box	GAAAAACAAG*T*AGG*T*GGC*A*GGAGGCTACG	CGTAGCCTCCT*G*CC*A*CCT*A*CTTGTTTTTC
SSeCKS-TK heterologous promoter[b]		
Minimal TK	CCGATGCTCGAGCAGCGTCTTGTCATTGGCGA	GGATAAAGCTTTTAAGCGGGTCGCTGCAGG
SSeCKS −106/−49	TCGATAGGTACCGAGCTCTTACGC	CCGATGCTCGAGAGCCACCTCGTAGCCTCC
RT-PCR		
SSeCKS α	AAGAATGGTCAGCTGTCTGC	TGACAGTGAGTAGCTGGACG
SSeCKS β	AGGAGAAGGAGACTTCCTGC	TGACAGTGAGTAGCTGGACG
SSeCKS α+β	TAATGGAAGTGGCCAGATGTC	TGCAATCTGCTTTGTCTTGG
β-Actin	TTCTTTGCAGCTCCTTCGTTGCCG	TGGATGGCTACGTACATGGCTGGG
MS Sequenom[c]		
SSeCKS α	TAATTAAGTGGAGGAAGAAAATAGATAGGT	AAAAATTCAAAAACAACAAAAAAAA

[a] Mutated nucleotides are indicated in italics.
[b] Restriction enzyme sites are underlined.
[c] primers used after bisulfite treatment.

Western blot analysis showed avian v-Src protein was specifically expressed in v-*Src*/3T3 cells (using mAb-EC10), resulting in dramatic increases in Src autophosphorylation (poY416) and total cellular tyrosine phosphorylation in v-*Src*/3T3 cells compared with the control NIH3T3 cells. This indicates that Src is constitutively activated in v-*Src*/3T3 cells (Fig. 1*C*). Consistent with our previous studies, the abundance of both SSeCKS α and β protein isoforms was decreased markedly in v-*Src*/3T3 cells, although both SSeCKS isoforms could be detected in v-*Src*/3T3 lysates after longer exposures as shown in Fig. 1*C*. Semi-quantitative RT-PCR analysis with isoform-specific primers (Table 1) showed that the down-regulated levels of SSeCKS α and β mRNAs (Fig. 1*D*) correlated with decreases in α and β protein levels in v-*Src*/3T3 cells (Fig. 1*C*). Similar decreases in SSeCKS protein and mRNA levels by v-*Src* were found in at least three independent v-*Src*/3T3 clones and one v-*Src*-transformed mouse embryonic fibroblast (v-*Src*/MEF) cell line (data not shown). We previously showed that v-*Src* decreased SSeCKS transcript *and* protein levels to similar extents, based on Northern and Western blots (1, 2, 25). Thus, SSeCKS abundance is likely controlled by v-*Src* at the level of transcription. However, for Fig. 1*D*, we chose PCR cycle numbers that allowed the simultaneous visualization of SSeCKS isoform transcript levels in NIH3T3 and v-*Src*/3T3 cells, conditions that will allow us in the experiments below to gauge treatments that could derepress SSeCKS transcription in v-*Src*/3T3 cells.

Decreased SSeCKS mRNA Steady-state Levels in v-Src/3T3 Cells Are Not Mediated by Alteration in mRNA Stabilities—Because mRNA steady-state levels can be controlled by both mRNA synthesis rate and post-transcriptional mRNA stability (mRNA degradation rate), we examined whether the v-*Src*-induced down-regulation of SSeCKS was because of changes in mRNA stability. NIH3T3 and v-*Src*/3T3 cells were treated with actinomycin D to inhibit transcriptional initiation, and then SSeCKS mRNA levels were determined at various time points by semi-quantitative RT-PCR. The PCR products were resolved on 2% agarose gel, visualized by ethidium bromide staining, and quantified by densitometry analysis (Fig. 2*A*). The mRNA decay slopes reveal that there was no significant difference in the degradation rates of either SSeCKS mRNA isoform in NIH3T3 cells *versus* v-*Src*/3T3 cells (Fig. 2*B*). Therefore, v-*Src*-induced down-regulation of SSeCKS mRNA was not mediated by decreasing mRNA stability, suggesting that SSeCKS message abundance is controlled by repression of promoter activity.

Mapping of Transcriptional Start Sites (TSS) of SSeCKS—As a first step toward to studying SSeCKS promoter control mechanisms, we mapped the SSeCKS TSS using primer extension analysis. As shown in Fig. 3, specific extension bands were detected for SSeCKS α or β isoforms, suggesting that each isoform has a single major TSS in NIH3T3 cells. The precise location of the TSS was obtained by running in parallel a sequencing reaction performed with the same primer used in the extension

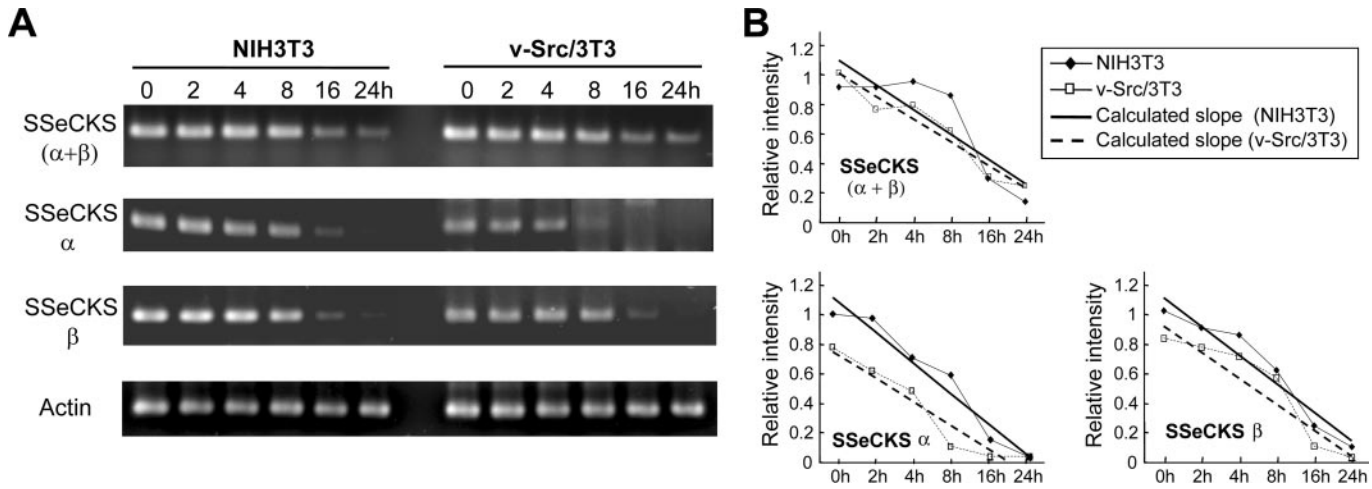


FIGURE 2. **v-*Src* does not alter SSeCKS mRNA stability.** *A*, total RNA was extracted from NIH3T3 or v-*Src*/3T3 cells following actinomycin D (5 μg/ml) treatment for the times indicated and analyzed for SSeCKS mRNA levels by RT-PCR. *B*, SSeCKS mRNA levels were determined by densitometric analysis and were plotted as decay curves. Because of the lower level of SSeCKS mRNA in v-*Src*/3T3 relative to NIH3T3 cells, more PCR cycles were used for v-*Src*/3T3 cells (see "Experimental Procedures") to get comparable amplifications. Calculated slopes were shown for comparison in the SSeCKS mRNA degradation rates between NIH3T3 and v-*Src*/3T3 cells.

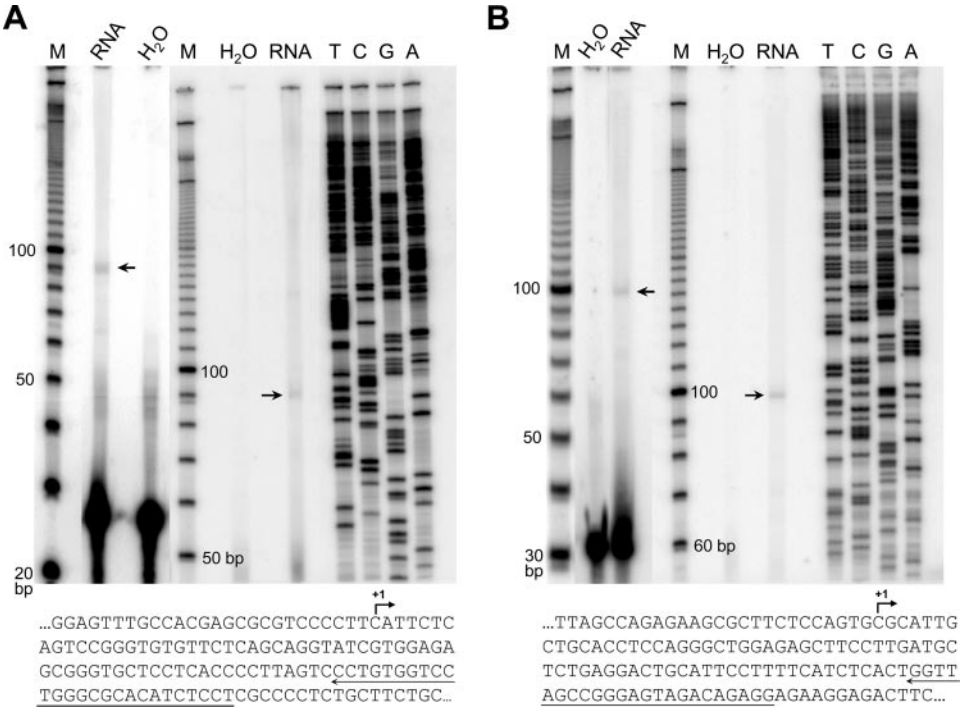


FIGURE 3. **Mapping of the SSeCKS α and β TSS in NIH3T3 cells by primer extension analysis.** Total RNA (50 μg) isolated from NIH3T3 cells, or an equal volume of H_2O as a control, was hybridized to a ^{32}P-end-labeled antisense oligonucleotide primer specific to SSeCKS α (*A*) or β (*B*) mRNA. The annealed primers were elongated with reverse transcriptase and then resolved on a denaturing 8% polyacrylamide gel along with a 10-bp DNA marker (*M*) and sequencing ladders. The annealed sequences of each primer used in the assay are *underlined*, and the nucleotide corresponding to the TSS is designated as +1. *Arrows,* major extension bands.

assay. The nucleotide C in the TTCAT motif was defined as the TSS (designated +1) of the SSeCKS α isoform (Fig. 3*A*), and the nucleotide C in the GTGCG motif was identified as the TSS of the β isoform (Fig. 3*B*). The start sites identified here are very close to those identified by Streb *et al.* (18) in rat smooth muscle cells (3 bp downstream for α and 8 bp downstream for β) using a 5′-rapid amplification of cDNA ends technique.

Identification of VSRE in SSeCKS Promoters—Roughly 5 kb of promoter region upstream of the TSS of each isoform (−4920/+36 for isoform α and −4758/+119 for isoform β) was cloned into the pGL3-Basic luciferase reporter vector, and in order to locate the VSRE, we generated a series of progressive promoter deletion mutants. These full-length promoter and deletion constructs were then transiently co-transfected into NIH3T3 and v-*Src*/3T3 cells along with a *Renilla* luciferase reporter driven by the TK promoter, representing a normalization control. As shown in Fig. 4*A*, the −4920/+36 SSeCKS α promoter sequence induced robust luciferase activities in both cells and, more importantly, exhibited considerably lower relative promoter activity in v-*Src*/3T3 cells. Truncation of the sequence between −4920 and −2677 enhanced promoter activities in both cells but did not affect the v-*Src* responsiveness, suggesting that this region contains v-*Src*-independent repressors. Deletions of the sequence between −2677 and −106 reduced the promoter activities in both cell types but still had no effect on the v-*Src*-mediated repression. However, truncation of the sequence between −106 and −26 abolished both VSR and basal promoter activities. These findings were observed in at least two independently derived v-*Src*/3T3 clones and another v-*Src*/MEF cell line (data not shown). These results demonstrate that the SSeCKS α proximal promoter region between −106 and −26 encodes the minimal VSRE and also contains the minimal *cis*-acting sequences required for basal promoter activity. Interestingly, a lucif-

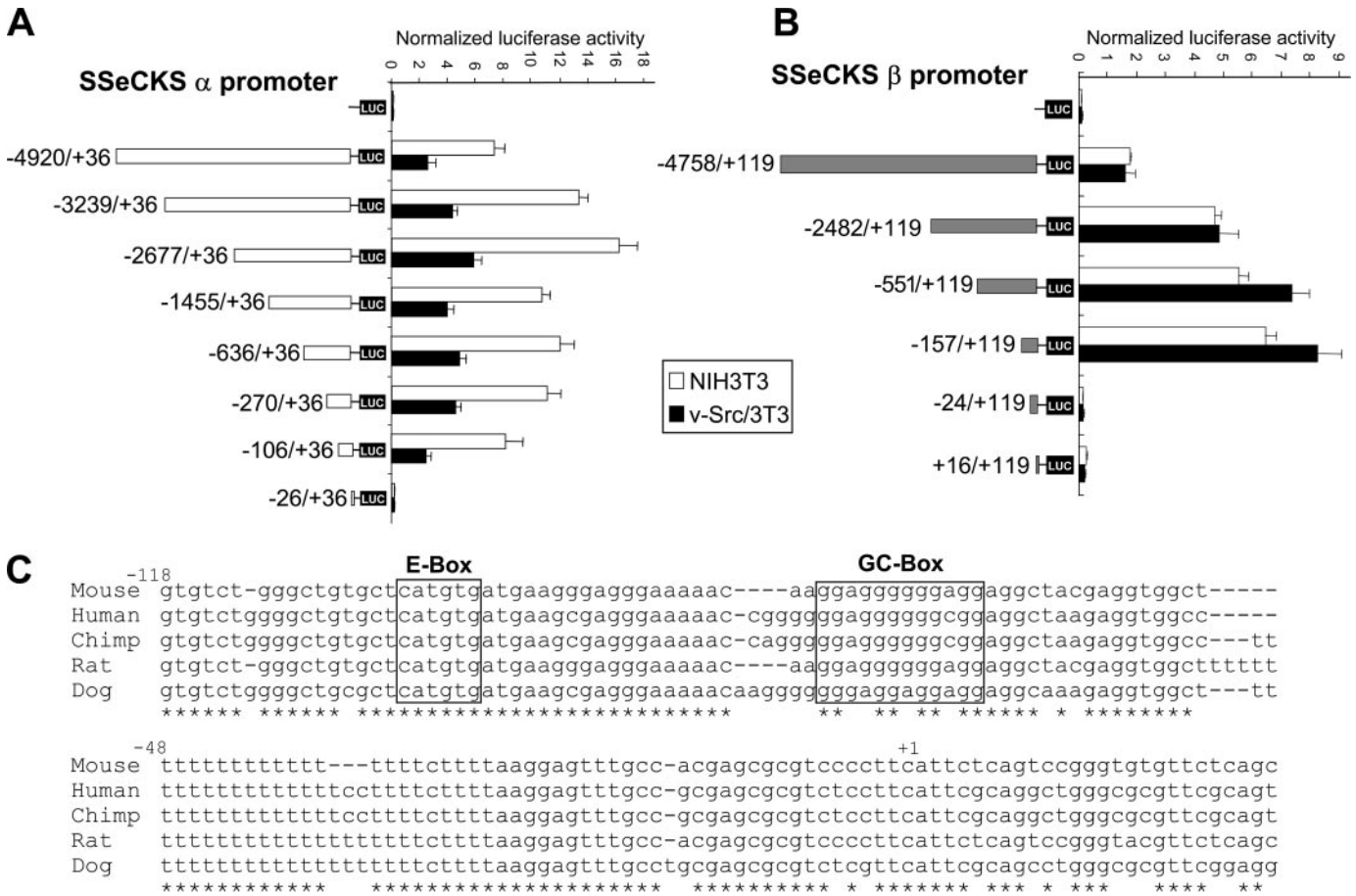


FIGURE 4. **Mapping of VSRE in SSeCKS α and β promoters.** *A* and *B*, NIH3T3 and v-*Src*/3T3 cells were transiently co-transfected with a deletion series of the SSeCKS α (*A*) and β promoter (*B*) sequences cloned into pGL3B-luciferase vectors along with pRL-TK (as a normalization control for transfection efficiency and cell proliferation rates). Cells lysates were produced after 48 h and then assayed for luciferase activity. The normalized luciferase value for each assay is shown as the mean of three replicates ± S.D. The representative result of three independent experiments is shown. *C*, DNA sequence alignment of the SSeCKS α proximal promoters from mouse, human, chimp, rat, and dog. *Asterisks* indicate the conserved nucleotides among all five species. Consensus E- and GC-boxes are *highlighted*.

erase-reporter construct containing roughly 5 kb of the human α SSeCKS (Gravin) promoter was down-regulated in LNCaP and C-42 prostate cancer cells compared with untransformed, immortalized P69SV40T human prostate epithelial cells (data not shown), suggesting that promoter sequences controlling down-regulation in cancer are also present in the human α SSeCKS allele.

To our surprise, neither the −4758/+119 promoter sequence of SSeCKS β isoform nor any of its deletion constructs exhibited v-*Src*-mediated repression in the luciferase assay, although the region proximal to the TSS (−157/+119) was sufficient to encode adequate promoter activity (Fig. 4*B*). As with the α promoter, the distal β promoter region from −4758 to −2482 seems to harbor v-*Src*-independent control sequences that repress the basal activity.

Sequence conservation across different species, especially in promoter regions, strongly suggests common regulatory mechanisms. We carried out a cross-species comparison of SSeCKS α proximal promoter sequences shown in Fig. 4*A* to be sufficient for VSR and basal promoter activity. As shown in Fig. 4*C*, the α proximal promoter sequence was highly conserved between mouse, human, chimp, rat, and dog, and moreover, there was equal E- and GC-box spacing relative to the TSS, further strengthening its functional importance in regulating gene expression in the context of the α promoter.

E- and GC-boxes in the SSeCKS α Proximal Promoter, Bound by USF1 and Sp1/3, Respectively, Are Crucial for the v-Src Responsiveness—To identify the precise regulatory elements responsible for VSR activity, we generated fine deletion constructs with the SSeCKS α proximal promoter and then performed luciferase assays. As shown in Fig. 5*A*, deletion of the region between −106 and −89, which contains the consensus E-box, markedly reduced promoter activities in both NIH3T3 and v-*Src*/3T3 cells and abrogated v-*Src* responsiveness. Further deletion of the region between −89 and −67, which contains a GC-box site, resulted in a similar decrease in the basal promoter activities. These results indicate that the E-box in the proximal promoter is critical for the v-*Src* responsiveness.

We investigated the transcriptional factors binding to the α proximal promoter by EMSA with three overlapping synthetic DNA oligonucleotides, spanning the sequence between −106 and −26 (Fig. 5*B*). Equal amounts of total nuclear extract (5 μg) prepared from NIH3T3 and v-*Src*/3T3 cells were used in each assay. As shown in Fig. 5*C*, oligo-1 (−106/−71) facilitated the formation of two DNA-protein complexes, C1 and C2, and two major binding complexes, C2 and C3, were formed to oligo-2

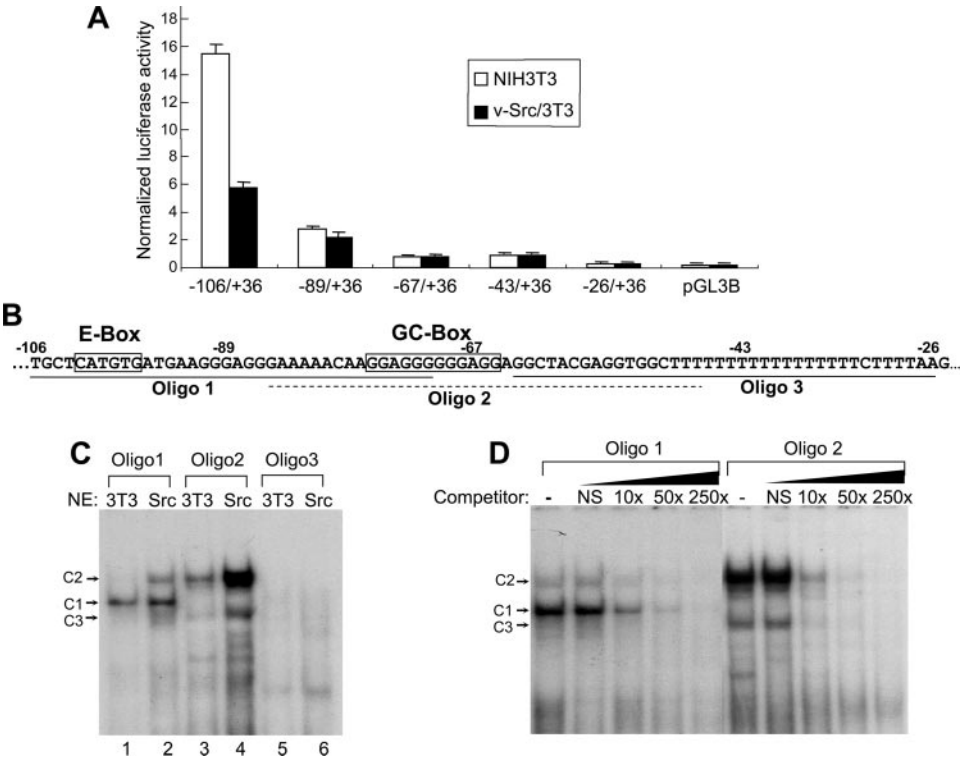


FIGURE 5. **VSRE are located in the α SSeCKS proximal promoter.** *A*, NIH3T3 or v-*Src*/3T3 cells were transfected with various SSeCKS α proximal promoter-luciferase reporter constructs along with pRL-TK, and lysates isolated after 48 h were assayed for luciferase activity. The normalized luciferase value for each assay is shown as the mean of three replicates ± S.D. The representative result of three independent experiments is shown. *B*, DNA sequence of the SSeCKS α proximal promoter from nucleotides −106 to −26. The three overlapping oligonucleotides spanning this region used in the EMSA are indicated by *solid* or *dotted lines*. *C*, EMSA analysis was carried out with ^{32}P-labeled oligonucleotides and nuclear extracts (*NE*) (5 μg/lane) prepared from NIH3T3 or v-*Src*/3T3 cells. The two DNA-protein complexes formed with oligo-1 were designated as C1 and C2, and the two major binding complexes formed with oligo-2 were designated as C2 and C3. *D*, nuclear extracts prepared from v-*Src*/3T3 cells were used in competition assays with molar excess of unlabeled nonspecific (*NS*) or specific oligonucleotides as indicated.

(−85/−47), but no significant gel shifts were detected using oligo-3 (−63/−26). All three DNA-protein complexes (C1, C2, and C3) were specific as demonstrated by competition experiments with molar excesses of specific and nonspecific unlabeled oligonucleotides (Fig. 5*D*). The binding activity of protein complex C1 to oligo-1 was comparable between the NIH3T3 and v-*Src*/3T3 cells (Fig. 5*C*, *lanes 1* and *2*). In contrast, the binding complexes of C2 and C3 were formed preferentially in v-*Src*/3T3 cells compared with NIH3T3 cells (Fig. 5*C*, *lanes 3* and *4*), suggesting that these two complexes correlate with v-*Src* responsiveness. These findings were also observed using nuclear extract prepared from another independent v-*Src*/3T3 clone (data not shown). The presence of C1 complex solely in oligo-1 but not in oligo-2 strongly suggested that the binding site of C1 was in the nonoverlapping region of oligo-1 (*e.g.* the E-box). Given that C2 co-migrated with either oligo-1 or -2, but that it showed stronger binding with oligo-2, it is possible that the C2 binding is at the overlap of oligo-1 and -2 (*e.g.* the GC-box). To test whether the binding site of C1 is the E-box, and C2 is the GC-box, we performed EMSAs with oligo-4 and oligo-4m (Fig. 6*A*), versions of oligo-1 lacking the GC-box site at its 3′-end or encoding a mutated E-box site. As shown in Fig. 6*B*, oligo-4 failed to induce the formation of complex C2, although it had no effect on the formation of complex C1 (*lanes 3* and *4*), strongly suggesting that the binding site of C2 induced by oligo-1 was at the GC-box. Mutation of the E-box in oligo-4m completely abrogated the formation of complex C1 (Fig. 6*B*, *lanes 5* and *6*), strongly suggesting that the protein-DNA interaction site of C1 was at the E-box. In addition, mutation of the GC box in oligo-2 (oligo-2m) abolished the formation of both complex C2 and C3 (Fig. 6*B*, *lanes 9* and *10*), showing that the DNA-protein interaction site of complex C2 or C3 with oligo-2 was likely at the GC-box.

The c-Myc- and c-Myc-related family of proteins, such as USF1 and USF2, is among the helix-loop-helix transcriptional factors known to bind E-box sequences (26, 27). Moreover, it has been reported that USF1 binds to the E-box on α SSeCKS promoter in Rat-2 fibroblasts (18). To determine whether c-Myc or USF1 was the E-box-binding protein that forms the C1 complex, we performed supershift EMSAs with antibodies (Ab) specific for c-Myc or USF1. Addition of the anti-USF1 Ab supershifted the C1 complex (Fig. 6*B*, *ss*), whereas preimmune IgG control had no effect on the migration of C1 (Fig. 6*B*, *lanes 13* and *14*). In contrast, Ab against c-Myc failed to either compete or supershift the C1 complex (data not shown). These data indicate that USF1, but not c-Myc, binds *in vitro* to the SSeCKS α proximal promoter at the E-box.

GC-boxes are known to be bound by the Sp and Krüppel-like factor families of transcription factors (28). We then examined whether Sp1 or Sp3 were present in the binding complexes, C2 and C3. Upon more careful resolution, the thick band of C2 formed with oligo-2 in EMSA (Fig. 5*C*, *lanes 3* and *4*) can be separated into several complexes as follows: a doublet, designated C2a and C2b, and a faster migration C2c (Fig. 6*B*, *lanes 15* and *16*). Addition of anti-Sp1 Ab led to a supershift of C2a but had little effect on C2b, C2c, or C3 (Fig. 6*B*, *lane 17*), whereas anti-Sp3 Ab resulted in supershift of C2b and of the entire C3 (Fig. 6*B*, *lane 18*). Neither Ab affected the migration of C2c. Addition of both Abs supershifted C2a, C2b, and C3 but still had little effect on C2c (Fig. 6*B*, *lane 19*). These results strongly suggest that the DNA-protein complexes formed at the GC-box primarily contain Sp1 and Sp3; whether C2c represents another Sp family member or an additional non-Sp complex or nonspecific binding is still unclear. It should be noted that the C2c complex is also formed preferentially in v-*Src*/3T3 cells. In addition, a ChIP assay confirmed the EMSA findings, namely that binding to the SSeCKS α proximal promoter by Sp1 and

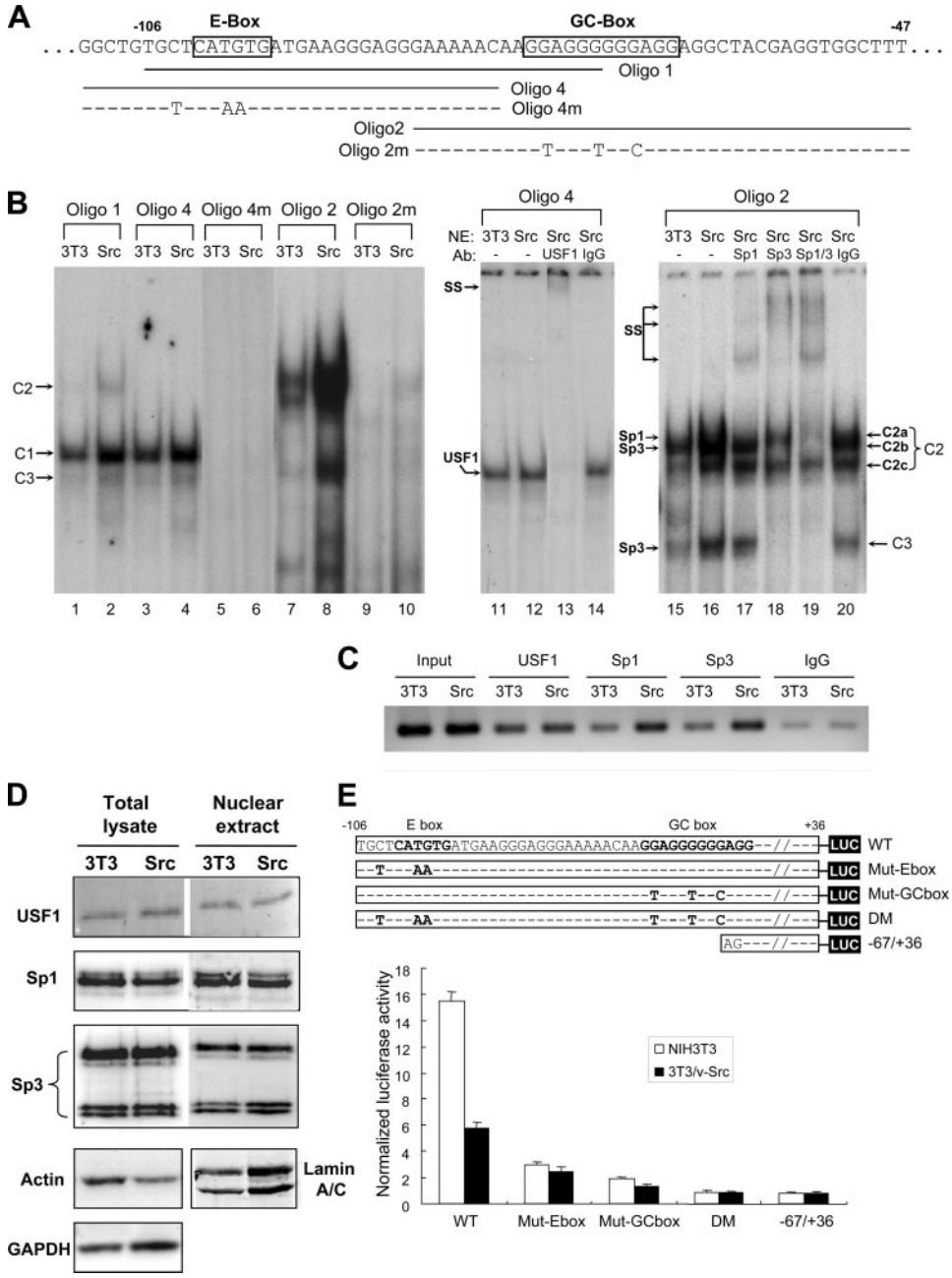


FIGURE 6. **E- and GC-boxes in the SSeCKS α proximal promoter, bound by USF1 and Sp1/3, respectively, are crucial for VSR activity.** *A*, sequences of the five oligonucleotides used in the EMSA, containing wild type E- or GC-boxes (oligo 1, -2, and -4) or mutated E- or GC-boxes (oligo-2m, -4m; only the mutated nucleotides are shown). *B*, EMSA analysis using ^{32}P-labeled oligonucleotides shown in *A*. Supershift experiments were carried out by adding antibodies specific for USF1, Sp1, and/or Sp3 (or IgG controls), as indicated. *SS*, supershift. *C*, ChIP assay. NIH3T3 or v-*Src*/3T3 cells were treated with formaldehyde, lysed, and subjected to immunoprecipitation with antibodies specific for USF1, Sp1, Sp3, or IgG control. SSeCKS α proximal promoter sequences (-270 to $+33$) were amplified by PCR from the immunoprecipitates. *D*, whole cell lysates or nuclear extracts prepared from NIH3T3 or v-*Src*/3T3 cells were immunoblotted with antibodies specific for USF1, Sp1, or Sp3. Actin and GAPDH blots are shown as loading controls for whole cell lysates; lamin A/C is a marker of nuclear preparation. Note that v-*Src* typically decreases actin 2-fold, increases GAPDH 2-fold, and increases lamin A/C 2–3-fold (Y. Bu and I. H. Gelman, unpublished observations); and thus, protein-loading normalization usually requires analyzing several proteins. *E*, NIH3T3 or v-*Src*/3T3 cells were co-transfected with the wild type $-106/+36$ SSeCKS α promoter/luciferase construct or similar reporter constructs containing mutated E- or GC-boxes, or both (*top panel*), along with pRL-TK. Cell lysates were harvested after 48 h and assayed for luciferase activity (*bottom panel*). The normalized luciferase value for each assay is shown as the mean of three replicates $\pm$ S.D. The representative result of three independent experiments is shown.

Sp3, but not by USF1, is enhanced in v-*Src*/3T3 cells compared with NIH3T3 cells (Fig. 6*C*). Importantly, the increased binding of Sp1 and Sp3 to the GC box in v-*Src*/3T3 cells was not because of either increased total protein expression or increased nuclear localization of these proteins in v-*Src*/3T3 cells (Fig. 6*D*). The lamin A/C protein level is used merely as a marker of nuclear preparation; it cannot be used as a loading control because its relative abundance is altered by v-*Src* (as is true for many other typical loading control proteins).[3]

We next investigated the functional importance of E- and GC-boxes in mediating v-*Src* responsiveness. Thus, proximal α promoter-luciferase constructs were produced that incorporate the mutations in oligo-4m (loss of USF1 binding), oligo-2m (loss of Sp1/3 binding), or both (Fig. 6*E*). Loss of either USF1 or Sp1/3 binding ablated both VSR and basal promoter activities, and mutation to both boxes (DM) decreased these activities roughly 50% more (Fig. 6*E*). Taken together with the increased binding of Sp1/3 to the GC-box in v-*Src*/3T3 cells (Fig. 6*B*), these data strongly suggest that Sp1/3 encode dual regulatory roles for this promoter as follows: as inducers of basal promoter activity in both untransformed and v-*Src*-transformed cells and as repressors of promoter activity in v-*Src*/3T3 cells.

Although the β proximal promoter also contains E- and GC-boxes spaced similarly upstream of the TSS as in the α promoter, these boxes seem not to be sufficient for the VSR activity in the β promoter. This suggests that VSR activity is governed by E-/GC-box spacing constraints and/or by the E-/GC-boxes plus other sequences found only in the α proximal promoter.

The SSeCKS α Proximal Promoter Is Sufficient to Confer VSR to a Heterologous Promoter—To test whether the SSeCKS α proximal sequence between -106 and -49, containing E- and GC-boxes, is sufficient to confer VSR activity, this sequence was spliced immediately upstream of a minimal TK promoter driving firefly luciferase.

[3] I. H. Gelman, unpublished observations.

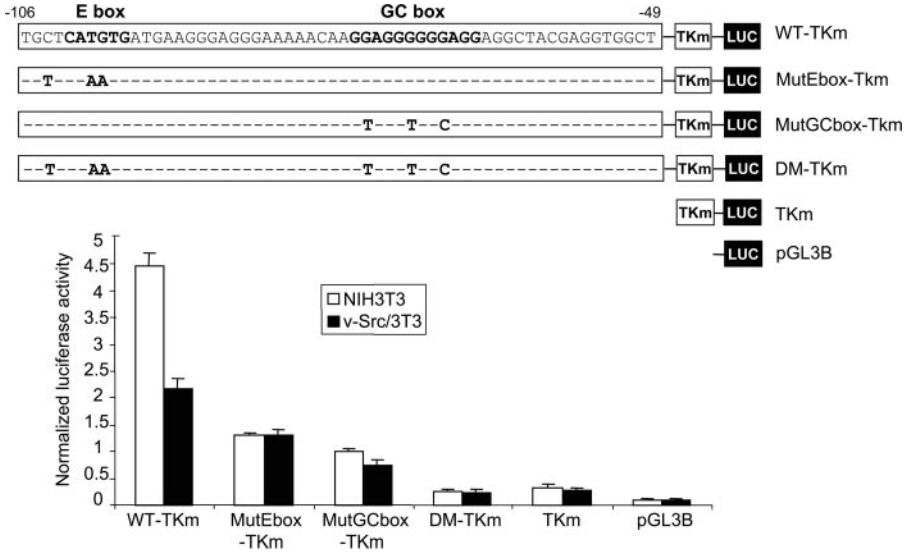


FIGURE 7. **The SSeCKS α proximal promoter is sufficient to confer VSR activity to a heterologous TK promoter.** Either the wild type SSeCKS α proximal promoter (−106 to −49), or those containing E- and/or GC-box mutations (*top panel*) were fused upstream of the minimal TK promoter driving a luciferase reporter and used to co-transfect NIH3T3 or v-*Src*/3T3 cells along with pRL-TK control plasmid. Cell lysates were prepared after 48 h and assayed for luciferase activity. The normalized luciferase value for each assay is shown as the mean of three replicates ± S.D. Data shown are representative of three independent experiments.

As shown in Fig. 7, this 58-bp sequence was sufficient to induce VSR to the heterologous TK promoter. Moreover, mutation of either the E- or GC-box in this sequence abrogated its ability to confer VSR. Collectively, therefore, the E- and GC-boxes in the context of the SSeCKS α proximal promoter were both necessary and sufficient for VSR activity.

Effects of Sp1 and/or Sp3 Overexpression on SSeCKS α Proximal Promoter Activity in NIH3T3 and v-Src/3T3 Cells—Given that the GC-box supports VSR activity and that increased Sp1/Sp3 binding to the GC-box was observed in EMSA using v-*Src*/3T3 lysates, we examined whether overexpression of Sp1 and/or Sp3 was sufficient for transcriptional repression in NIH3T3 and v-*Src*/3T3 cells. Thus, we co-transfected increasing amounts of Sp1 or Sp3 expression plasmid (from 0.01 to 0.4 μg) with a set amount of the −106/+36 α promoter construct. As shown in Fig. 8*A*, overexpression of Sp1 led to a dose-dependent increase in promoter activity in both cells. In contrast, only high overexpression levels of Sp3 increased promoter activity in NIH3T3 cells but had little effect in the v-*Src*/3T3 cells (Fig. 8*B*). Because Sp3 can exert transcriptional inhibition by competitively antagonizing the action of Sp1 (29), and sumoylation of Sp3 potentiates its repressive activity (30), we reasoned that the failure of Sp3 to repress the SSeCKS α promoter activity could be because of limited sumoylation of the exogenous Sp3. However, inclusion of expression vectors for SUMO and for the PIAS1 ubiquitin-protein isopeptide ligase-3, required to conjugate SUMO to Sp3 (31), led to increased promoter activity in both cells, albeit to a much lesser extent in v-*Src*/3T3 cells (Fig. 8*C*). In contrast, increasing levels of Sp3 (0.1–0.4 μg) co-transfected with a set level of Sp1 (0.1 μg) resulted in the reduction of the Sp1-mediated transactivation in a dose-dependent manner in v-*Src*/3T3 cells but not in NIH3T3 cells (Fig. 8*D*). The exogenous expression of the Sp1, Sp3, PIAS1, and SUMO proteins was confirmed by immunoblotting (supplemental Fig. 1). Taken together, these results indicate that Sp1 alone is a strong activator of the SSeCKS α promoter in both NIH3T3 and v-*Src*/3T3 cells, whereas Sp3 is a weak activator alone in both cells, but in the presence of both Sp1 and Sp3, Sp3 can antagonize the Sp1-mediated transactivation of α promoter activity only in v-*Src*/3T3 cells. This suggests that v-*Src* converts the Sp1/3 complex from an activator to a repressor, possible through post-translational modifications or through the induction of co-repressors.

VSR Activity Correlates with Changes in Chromatin Structure—The transient luciferase assay (Fig. 4*A*) only partly recapitulated the repression of SSeCKS transcripts at the endogenous level (Fig. 1*D*). This suggests that VSR activity of the endogenous α promoter may also be controlled by epigenetic mechanisms, such as DNA methylation or changes in chromatin structure, that are not manifest on exogenous reporter plasmids during a transient expression assay. Therefore, we examined whether v-*Src*-mediated down-regulation of SSeCKS transcription was controlled by epigenetic mechanisms. Indeed, previous reports indicated that AKAP12/Gravin is inactivated by promoter hypermethylation in gastric and colon cancer (12, 32). RT-PCR analysis showed that treatment of v-*Src*/3T3 cells with the DNA methyltransferase inhibitor, 5-aza-C, failed to restore the steady mRNA levels of either isoform of SSeCKS (Fig. 9*A*, *lanes 3* and *4*), indicating that DNA methylation was not involved in the v-*Src*-mediated down-regulation of SSeCKS. Moreover, we examined the methylation status of CpG islands in the SSeCKS proximal promoter sequences of the α isoform (see Table 1 for primers) by a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry approach (33) (Sequenom MassARRAY, RPCI Microarray and Genomics Core Facility), and we found no significant differences between control NIH3T3 and v-*Src*/3T3 cells (data not shown). In contrast, inhibition of histone deacetylase (HDAC) activities by treatment with TSA partly restored the steady mRNA level of the α isoform and fully restored β isoform in v-*Src*/3T3 cells (Fig. 9*A*, *lanes 5* and *6*), whereas combining with 5-aza-C treatment had little additive effect (Fig. 9*A*, *lanes 7* and *8*). Similar data were also found in another independent v-*Src*/3T3 clone and a v-*Src*/MEF cell line (data not shown). These data suggest that histone deacetylation, but not DNA methylation, plays a role in v-*Src*-mediated down-regulation of SSeCKS. This finding agrees with Rombouts *et al.* (34) who showed that TSA could derepress SSeCKS in a model of hepatic injury.

Because TSA treatment is known to affect the expression of a wide range of genes, the derepression of SSeCKS in

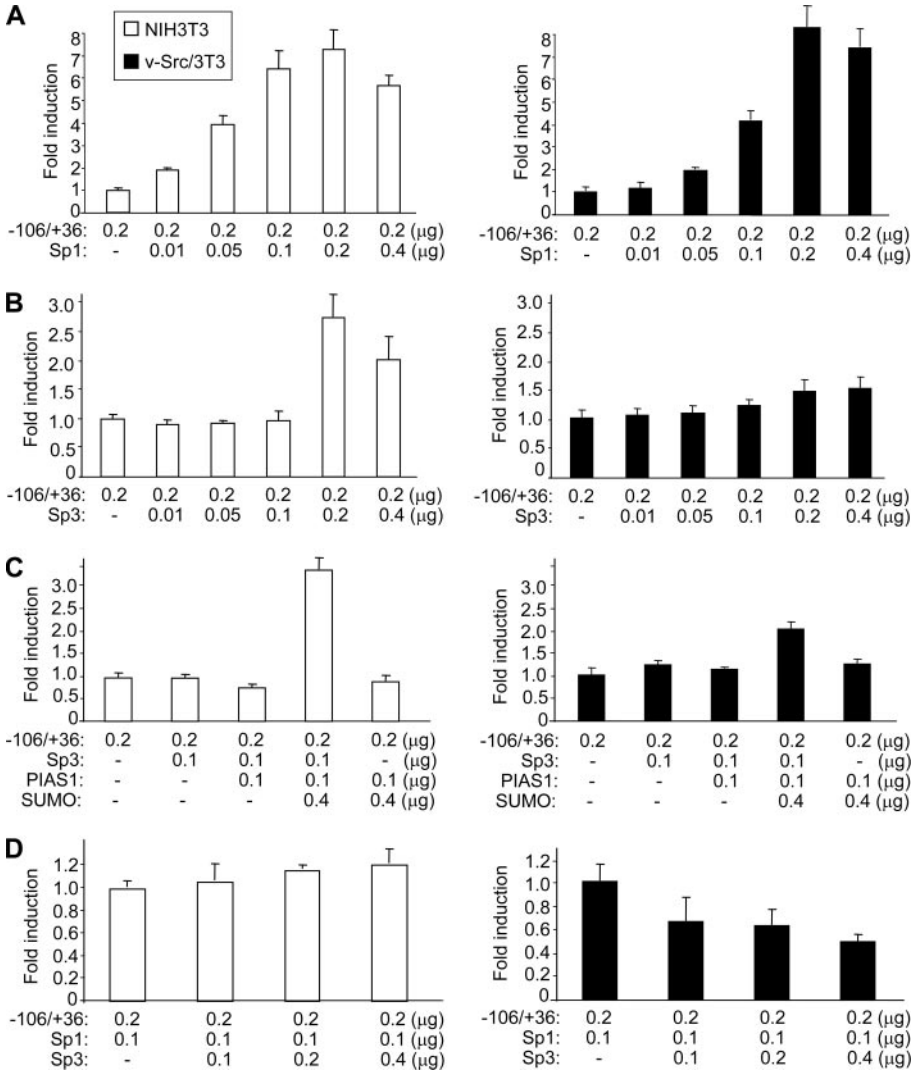


FIGURE 8. **Effects of overexpression of Sp1 and/or Sp3 on SSeCKS α proximal promoter activity in NIH3T3 and v-*Src*/3T3 cells.** *A* and *B*, effect of Sp1 (*A*) or Sp3 (*B*) on SSeCKS α proximal promoter activity, as determined by luciferase assay. NIH3T3 (*white bars*) or v-*Src*/3T3 cells (*black bars*) were transfected with the SSeCKS α proximal promoter-luciferase alone or with varying amounts of Sp1 (*A*) or Sp3 (*B*) expression plasmids as indicated. Cell lysates were prepared after 48 h and assayed for luciferase activity. *C*, effect of Sp3 sumoylation on the α proximal promoter. The SSeCKS α proximal promoter-luciferase construct was transfected into NIH3T3 or v-*Src*/3T3 cells alone or in combination with expression plasmids for Sp3, the PIAS1 ubiquitin-protein isopeptide ligase-3, and SUMO-1, as indicated. *D*, effect of Sp3 on the Sp1-mediated activation of the α proximal promoter. The SSeCKS α proximal promoter-luciferase construct was co-transfected into NIH3T3 or v-*Src*/3T3 cells with 0.1 μg of Sp1 expression plasmid plus varying amounts of Sp3 expression plasmid, as indicated. For all assays, the luciferase activity values are expressed as fold induction of the reporter alone, arbitrarily assigned a value of 1. Each *bar* is the mean of three replicates ± S.D. Data shown are representative of three independent experiments.

v-*Src*/3T3 cells induced by TSA could be indirect, *i.e.* not because of a direct increase in acetylated histones on the SSeCKS promoter. To test this possibility, we performed ChIP assays to examine the acetylation status of histone H3 and histone H4 markers, often associated with a more transcriptionally active chromatin structure (35, 36), on the SSeCKS proximal promoters in NIH3T3 and v-*Src*/3T3 cells. As shown in Fig. 9*B*, the degree of Ac-H3 and Ac-H4 binding to the SSeCKS α promoter was lower in v-*Src*/3T3 than that in the control NIH3T3 cells. Moreover, TSA treatment increased the binding of Ac-H3 and Ac-H4 in the v-*Src*/3T3 cells, showing that the derepression of SSeCKS α isoform by TSA in v-*Src* transformed cells is controlled by an increase in histone acetylation levels. In contrast, there was no difference in Ac-H3 and Ac-H4 binding levels on the proximal β promoter between the two cells, and correspondingly, TSA treatment did not alter Ac-H3 and Ac-H4 binding levels in v-*Src*/3T3 cells. This lack of change to the chromatinization of the β promoter contrasts with the finding in Fig. 9*A* that TSA strongly derepressed the transcription of β-SSeCKS in v-*Src*/3T3 cells, yet it agrees with our finding that the exogenously expressed β promoter failed to be down-regulated in v-*Src*/3T3 cells (Fig. 4*B*). These data suggest that the repression of β isoform may not be controlled by its own promoter but rather through a coordinate regulation of chromatin structure at the α promoter 68 kb upstream. Indeed, Streb and Miano (37) show evidence that the serum responsiveness of the β promoter could be controlled by the CArG box found in the α promoter. It cannot be ruled out, however, that the TSA-induced derepression of the β isoform was caused by the induction of transcription activators specific for the β promoter.

Given that hypo-acetylation of histones contributed to the VSR activity of the α promoter in v-*Src*/3T3 cells, we examined whether this could be due to changes in the abundance of HDAC1, -2, or -3. As shown in Fig. 9*C*, HDAC1 protein levels were increased in either whole cell lysates or nuclear extracts from v-*Src*/3T3 cells, whereas no significant differences in HDAC2 and HDAC3 levels were observed. Given that Sp1 and Sp3 can directly interact with HDAC1 (38–40) and that more Sp1/Sp3 bound to the GC-box on the SSeCKS α proximal promoter in v-*Src*/3T3 cells (Fig. 6*B*), we examined whether there is increased recruitment of HDAC1 to the α proximal promoter in v-*Src*/3T3 cells by performing a DNA affinity precipitation assay. As predicted, the oligonucleotides containing both E- and GC-boxes (−106/−47) and the one containing only the nonmutated GC-box (−85/−47 WT) pulled down an increased amount of HDAC1 from v-*Src*/3T3 lysates compared with lysates from NIH3T3 cells (Fig. 9*D*). Moreover, mutation in the GC-box significantly reduced its ability to interact with HDAC1 in both cells, indicating the HDAC1 binding to the SSeCKS α proximal promoter is

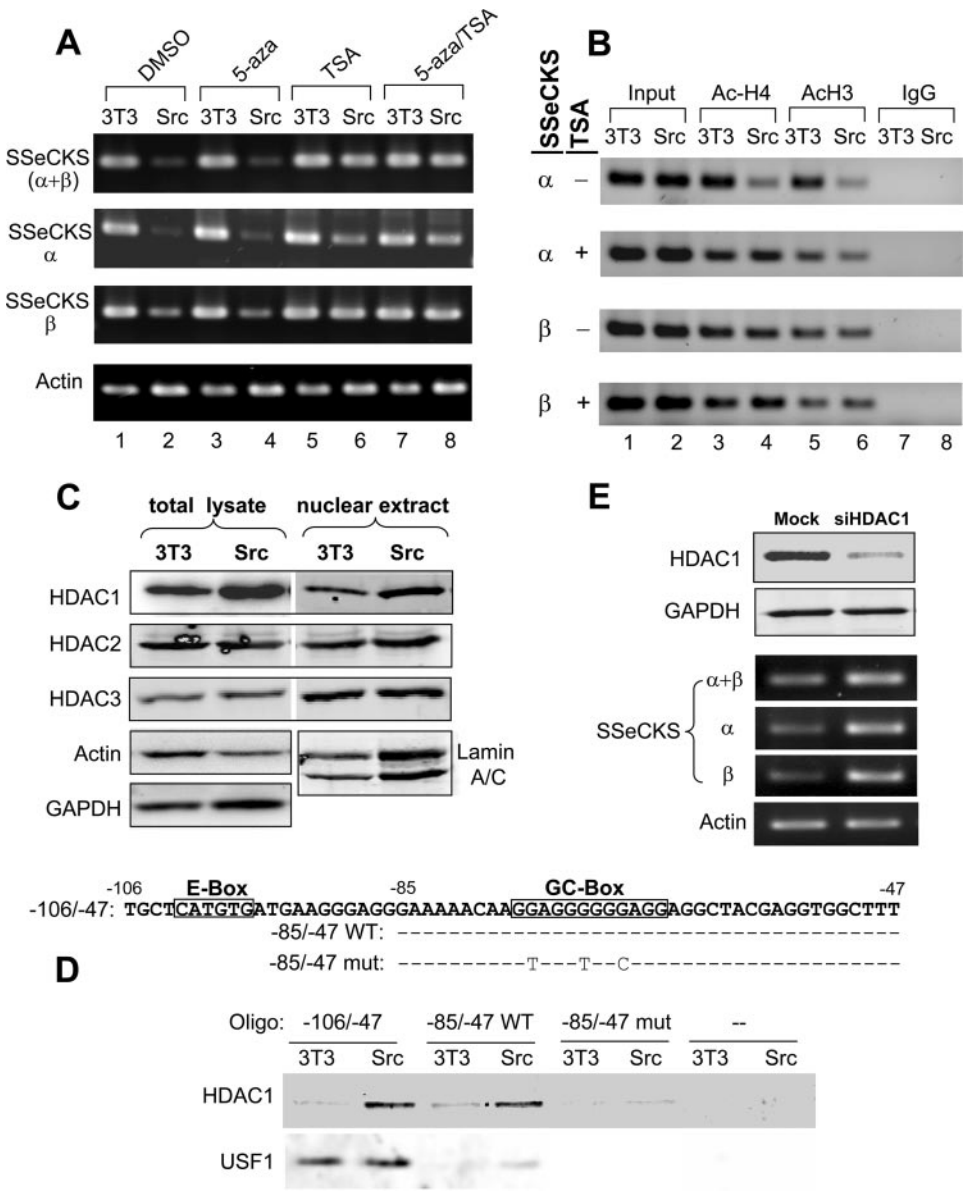


FIGURE 9. **Histone deacetylation is involved in the repression of SSeCKS transcription in v-*Src*/3T3 cells.** *A*, total RNA was isolated from NIH3T3 or v-*Src*/3T3 cells following treatment with vehicle (*DMSO*) or the fresh DNA methylation inhibitor 5-aza-C (500 nM for 72 h), and/or the histone deacetylase inhibitor TSA (330 nM for 24 h) as indicated and analyzed for SSeCKS mRNA levels by semi-quantitative RT-PCR. *B*, ChIP assay. After treating NIH3T3 or v-*Src*/3T3 cells with vehicle (*DMSO*) or TSA (330 nM for 24 h), chromatin was cross-linked by formaldehyde and then subjected to immunoprecipitation with antibodies specific for Ac-H3 or Ac-H4 as described under "Experimental Procedures." SSeCKS proximal promoter sequences (−270 to +33 for α, −248 to +43 for β) were amplified by PCR from the immunoprecipitates. *C*, whole cell lysates or nuclear extracts prepared from NIH3T3 and v-*Src*/3T3 cells were immunoblotted with antibodies against HDAC1, HDAC2, and HDAC3. Actin and GAPDH blots are shown as loading controls for whole cell lysates; lamin A/C is a marker of nuclear preparation. *D*, nuclear extracts (100 μg) from NIH3T3 or v-*Src*/3T3 cells were incubated with streptavidin-agarose beads coupled with or without biotin-labeled oligonucleotides containing SSeCKS α proximal promoter sequences (WT −106 to −47, WT −85 to −47, or −85 to −47 with a mutated GC-box). The precipitated protein complexes were immunoblotted with antibodies specific for HDAC1 or USF1. *E*, v-*Src*/3T3 cells were transfected with siRNA-HDAC1 (200 nM) or untransfected (mock) for 72 h. HDAC1 protein level was determined using Western blot analysis and GAPDH was the loading control. SSeCKS mRNA levels were analyzed by semi-quantitative RT-PCR using isoform-specific or common α/β primers.

dependent on the GC-box. We attempted to perform a ChIP assay to confirm these findings from the DNA affinity precipitation assay, but we were not able to detect any significant chromatin precipitation after normalization with the IgG control using the available HDAC1 antibody. However, the RNA interference-mediated knockdown of HDAC1 expression in v-*Src*/3T3 cells resulted in significant increases in SSeCKS mRNA levels of both isoforms (Fig. 9*E*), indicating that the co-repressor HDAC1 is involved in the transcriptional repression of SSeCKS in v-*Src* transformed cells. Taken together, these results suggest that VSR activity is mediated by the increased recruitment of the HDAC1 co-repressor via the enhanced binding of Sp1/Sp3 to the GC-box on the SSeCKS α proximal promoter.

Sp1/3 Potentiates the Repressive Activity of HDAC1 on the α Promoter—To support the notion that the recruitment of HDAC1 by Sp1/3 to the SSeCKS α promoter is involved in VSR activity, we co-transfected the −106/+36 reporter construct (0.2 μg) with increasing amounts of an HDAC1 expression plasmid (0.1–0.4 μg) alone or in combination with Sp1 and/or Sp3 expression plasmids (0.05 μg) into NIH3T3 or v-*Src*/3T3 cells. As shown in Fig. 10, the overexpression of HDAC1 alone led to a dose-dependent repression of the α proximal promoter activity in both NIH3T3 and v-*Src*/3T3 cells. The potency of this repression was ~2-fold higher in v-*Src*/3T3 cells, possibly because the enhanced binding of Sp1/Sp3 facilitated increased recruitment of HDAC1 to the α proximal promoter in the v-*Src*/3T3 cells. Moreover, inclusion of Sp1 or Sp3 potentiated the HDAC1-mediated repression in both cells; the combination of Sp1 and Sp3 had an effect, which was additive at best. Additionally, Sp3 consistently was a more potent co-repressor, especially at the higher concentrations of HDAC1. The exogenous expression of the HDAC1 protein was confirmed by immunoblotting (supplemental Fig. 1). These data strongly suggest that HDAC1 participates in VSR activity of the SSeCKS α promoter via an enhanced Sp1/Sp3 binding to the proximal promoter.

DISCUSSION

A wide range of genes have been shown to be up-regulated (41–45) or down-regulated (1, 46–51) in cells oncogenically transformed by v-*Src*. Some of these v-*Src* down-regulated

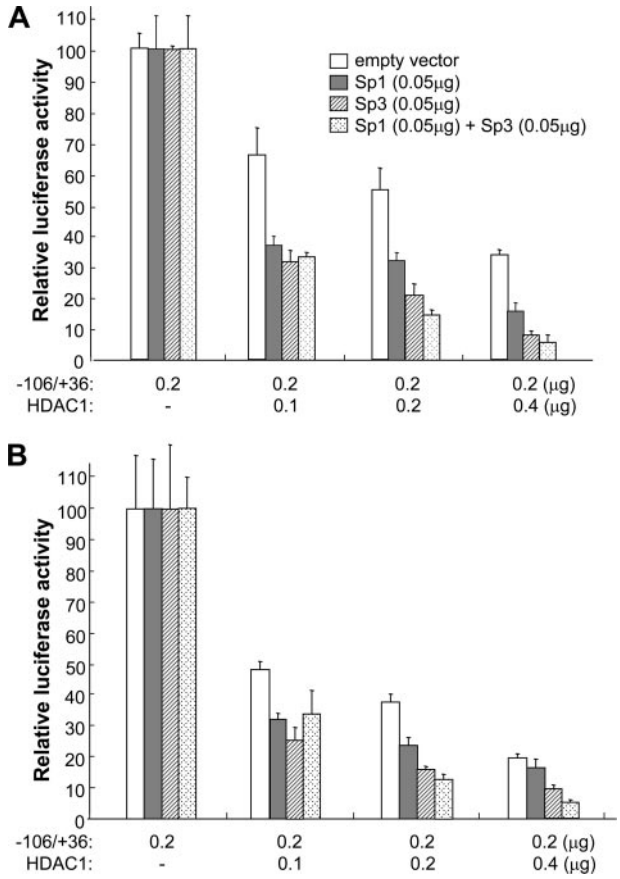


FIGURE 10. **HDAC1 combines with Sp1 and Sp3 to repress SSeCKS α proximal promoter activity in both NIH3T3 and v-*Src*/3T3 cells.** NIH3T3 cells (*A*) or v-*Src*/3T3 cells (*B*) were transfected with the SSeCKS α proximal promoter-luciferase construct alone or in combination with varying amounts of expression plasmid encoding HDAC1, with or without Sp1 and/or Sp3 expression plasmids as indicated. Cell lysates were prepared after 48 h and assayed for luciferase activity. The luciferase activity values for each assay are expressed as a percentage of those from the reporter alone, arbitrarily set at 100%. Each *bar* is the mean of three replicates ± S.D. Data shown are representative of three independent experiments.

genes, including *ssecks*, are recognized as tumor suppressor genes (1, 46, 52, 53). With the exception of studies on the *QR1* gene (50, 54), little is known regarding the mechanism by which v-*Src* down-regulates gene transcription. In this study, we used the *ssecks* gene as a prototype in order to explore the mechanisms involved in v-*Src*-mediated gene repression. We localize an essential VSRE to the E- and GC-boxes sites in the SSeCKS α proximal promoter. However, although these same elements (and similar relative spacing) are found in the SSeCKS β proximal promoter, neither the proximal nor 5-kb β promoter exhibited VSR activity. This could be due to the following: (i) the VSRE being >5 kb upstream in β promoter, (ii) the inactivity of the β promoter VSRE in transient expression assays, or (iii) a coordinated control of the β promoter by v-*Src* through VSRE found in the α promoter 68 kb upstream. The possibility that the transient expression assay fails to fully reflect VSR activity is borne out by our finding that even the v-*Src*-mediated down-regulation of the exogenous α promoter in the luciferase assays is roughly 2-fold lower than that of the endogenous α transcript levels (compare Fig. 1*D* to Fig. 4*A*). The notion that the upstream α promoter controls the VSR of the β promoter is supported by our findings that the β proximal promoter had similar levels of associated Ac-H3 and Ac-H4 in NIH3T3 and v-*Src*/3T3 cells, yet TSA treatment derepressed both α and β transcript levels in v-*Src*/3T3 cells. Thus, the ability of TSA to increase Ac-H3/Ac-H4 binding to the α proximal promoter strengthens our notion that v-*Src* affects chromatin structure at the upstream α promoter only.

Transcriptional repression can be mediated either by recruiting repressors or by releasing activators from the gene promoter. Our observation that increased Sp1/Sp3 binding to the GC-box in the α promoter in v-*Src*/3T3 cells, coupled with the fact that Sp1 can behave as either activator or repressor depending on the gene promoter context, strongly suggests that the repression of α-SSeCKS by v-*Src* is mediated by either the recruitment of repressors to the promoter or the post-translational modification of Sp1/Sp3. Indeed, our data indicate that Sp1 plays dual roles as follows: activating basal promoter activity in both NIH3T3 and v-*Src*/3T3 cells and repressing promoter activity in v-*Src*/3T3 cells. In addition, our overexpression experiments suggest that the increased Sp3 binding to the GC-box in α proximal promoter in v-*Src*/3T3 cells can antagonize Sp1-mediated transactivation in v-*Src*/3T3 cells. Indeed, Sp3 seems able to convert an apparent Sp1-containing complex into a repressor of the α proximal promoter in v-*Src*/3T3 but not in NIH3T3 cells.

Several studies have demonstrated post-translational modifications to Sp1/Sp3 as well as the induction of several Sp1/Sp3 partners in cancer cells. For example, the activation of the ERK MAPK by growth factors is known to induce Sp1 phosphorylation, correlating with increased DNA binding activity (55, 56). Other modifications such as acetylation occur in cancer cells,(reviewed in Ref. 57), although no specific modifications induced by Src have been described to date. Interestingly, Kuo *et al.* (58) showed that v-*Src* induced higher Sp1 binding activity to a GC-rich box in the proximal promoter of MMP-2 via an ERK-dependent pathway, but this correlated with induced MMP-2 expression.

In addition to the GC-box, our mutation assays indicate that the E-box is also crucial for VSR. Although USF1 was first identified as a transcriptional activator for the adenovirus late promoter (59), recent studies demonstrate that USF1 is also involved in transcriptional repression of certain genes (60–62). Moreover, Ge *et al.* (63) showed physical interaction between USF1 and Sp1, and at low Sp1 concentrations, Sp1 and USF1 could cooperatively transactivate the deoxycytidine kinase promoter, whereas at higher levels of Sp1, USF1 helps form a repressor complex. Therefore, although v-*Src* does not alter USF1 binding to the E-box on the α promoter, it is likely that the enhanced Sp1 binding to the adjacent GC-box in v-*Src*/3T3 cells converts the USF1 from a transcriptional activator to a repressor. Interestingly, we find that the USF1-Sp1-Sp3 complex seems more stable in v-*Src*/3T3 than in NIH3T3 cells because an oligonucleotide missing the proximal α promoter E-box (−87 to −47) can pull down USF1 in a GC-box-dependent manner (*i.e.* requiring Sp1/Sp3 binding) in v-*Src*/3T3 cells only (Fig. 9*D*). Taken together, the cooperative, and possibly physical, interactions between USF1 and Sp1 via binding to the

juxtaposed E- and GC-boxes on the SSeCKS α proximal promoter may represent a secondary mechanism involved in the v-*Src*-mediated repression in addition to the recruitment of HDAC co-repressors.

Several candidate co-repressors are induced in cancer cells, chief among them are the various HDACs, which we show play a significant role in the suppression of SSeCKS expression by v-*Src*. HDAC activity is reported to be increased in some tumors compared with normal tissues, and this increase has been associated with transcriptional repression of tumor suppressor genes (64, 65). We found that HDAC1 protein abundance is elevated in v-*Src*/3T3 cells compared with nontransformed NIH3T3 cells. Indeed, Src can phosphorylate HDAC3 (66), leading to increased enzymatic activity. Hsu *et al.* (67) showed that the *HER-2/neu* oncogene down-regulates the RECK metastasis-suppressor gene by inducing higher binding of an Sp1-HDAC1 complex to the RECK proximal promoter. Vitamin D_3 induces its cognate receptor to complex with Sp1 and HDAC1 in order to repress $p45^{Skp2}$ promoter activity in prostate cancer cells (68). Increased Sp1 binding to and recruitment of HDAC1 to a GC-box in the proximal promoter of the TGFβRII gene is required for transcriptional repression in pancreatic cancer (69). Other recent studies strengthen the notion that Sp1 can repress genes in cancer and in untransformed cells by recruiting HDACs (38, 39, 70, 71, 72).

The ability of Sp1 to recruit HDAC1 into gene repressor complexes correlates with our findings as follows: (i) TSA, but not 5-aza-C, derepresses both α and β SSeCKS transcription; (ii) decreases binding of Ac-H3 and Ac-H4 to the α promoter as shown in ChIP assays; (iii) v-*Src*/3T3 cells have 2–3-fold higher HDAC1 levels compared with NIH3T3; (iv) oligonucleotides encoding the proximal α promoter GC-box can pull down more HDAC1 in v-*Src*/3T3 than in NIH3T3 cells; and (v) RNA interference-mediated down-regulation of HDAC1 in v-*Src*/3T3 cells increases the steady-state mRNA levels of both SSeCKS isoforms. Thus, v-*Src* alters the chromatinization of the α SSeCKS promoter most likely by facilitating the formation of a repressor complex containing USF1, Sp1, Sp3, and HDAC1 that binds to proximal promoter sites.

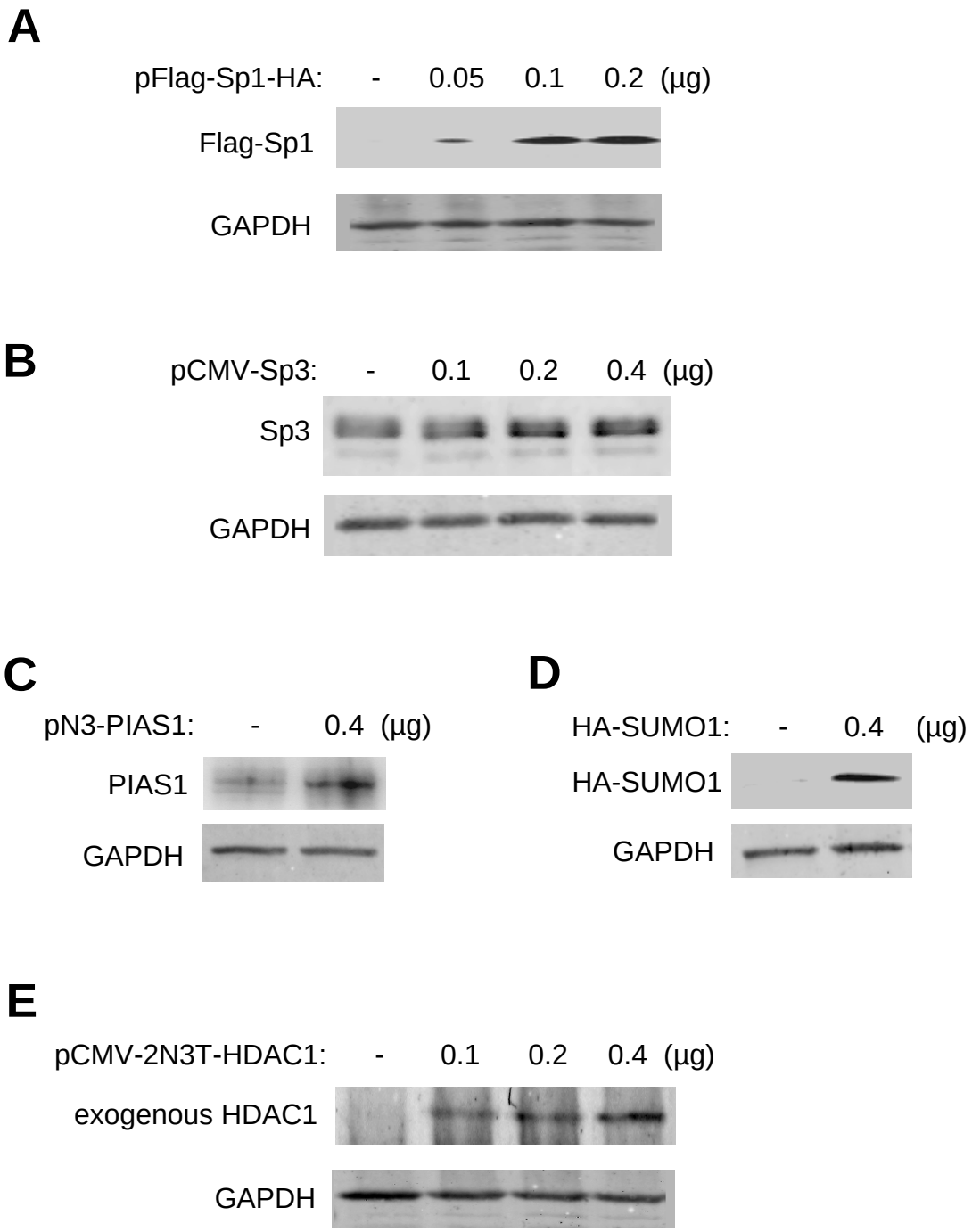
In this study, we identified *ssecks* as a cancer-related gene that can be up-regulated by the HDAC inhibitor, TSA. Moreover, we found that TSA can also reactivate the expression of human *ssecks* orthologue, *Gravin*, in prostate cancer cell lines, such as LNCaP and C4-2 (data not shown). Given the major focus on developing histone deacetylase inhibitors as clinical treatments for cancer (65), and with growing evidence that SSeCKS/Gravin/AKAP12 plays roles in the suppression of tumorigenesis and metastasis, it is interesting to speculate that derepression of SSeCKS might be an important mechanism by which the new generation of more selective HDAC inhibitors might mediate clinical cancer suppression.

Acknowledgments—We thank Adrian Black, Guntram Suske, Edward T. H. Yeh, and Didier Trouche for reagents; Andrei Bakin and Lionel Coignet for advice on experimental design; and Changhui Huang for help with constructing the SSeCKS promoter plasmids.

REFERENCES

1. Frankfort, B. J., and Gelman, I. H. (1995) *Biochem. Biophys. Res. Commun.* **206,** 916–926
2. Lin, X., Tombler, E., Nelson, P. J., Ross, M., and Gelman, I. H. (1996) *J. Biol. Chem.* **271,** 28,430–28,438
3. Nauert, J., Klauck, T., Langeberg, L. K., and Scott, J. D. (1997) *Curr. Biol.* **7,** 52–62
4. Gordon, T., Grove, B., Loftus, J. C., O'Toole, T., McMillan, R., Lindstrom, J., and Ginsberg, M. H. (1992) *J. Clin. Investig.* **90,** 992–999
5. Gelman, I. H. (2002) *Front. Biosci.* **7,** 1797
6. Lin, X., Nelson, P., and Gelman, I. H. (2000) *Mol. Cell. Biol.* **20,** 7259–7272
7. Gelman, I. H., Lee, K., Tombler, E., Gordon, R., and Lin, X. (1998) *Cell Motil. Cytoskeleton* **41,** 1–17
8. Xia, W., Unger, P., Miller, L., Nelson, J., and Gelman, I. H. (2001) *Cancer Res.* **15,** 5644–5651
9. Dhanasekaran, S. M., Barrette, T. R., Ghosh, D., Shah, R., Varambally, S., Kurachi, K., Pienta, K. J., Rubin, M. A., and Chinnaiyan, A. M. (2001) *Nature* **412,** 822–826
10. Perou, C. M., Sorlie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Rees, C. A., Pollack, J. R., Ross, D. T., Johnsen, H., Akslen, L. A., Fluge, O., Pergamenschikov, A., Williams, C., Zhu, S. X., Lonning, P. E., Borresen-Dale, A. L., Brown, P. O., and Botstein, D. (2000) *Nature* **406,** 747–752
11. Welsh, J. B., Zarrinkar, P. P., Sapinoso, L. M., Kern, S. G., Behling, C. A., Monk, B. J., Lockhart, D. J., Burger, R. A., and Hampton, G. M. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 1176–1181
12. Choi, M. C., Jong, H. S., Kim, T. Y., Song, S. H., Lee, D. S., Lee, J. W., Kim, T. Y., Kim, N. K., and Bang, Y. J. (2004) *Oncogene* **23,** 7095–7103
13. Garber, M. E., Troyanskaya, O. G., Schluens, K., Petersen, S., Thaesler, Z., Pacyna-Gengelbach, M., van de Rijn, M., Rosen, G. D., Perou, C. M., Whyte, R. I., Altman, R. B., Brown, P. O., Botstein, D., and Petersen, I. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 13784–13789
14. Wan, M., Sun, T., Vyas, R., Zheng, J., Granada, E., and Dubeau, L. (1999) *Oncogene* **18,** 1545–1551
15. Lin, X., and Gelman, I. H. (1997) *Cancer Res.* **57,** 2304–2312
16. Gelman, I. H., and Gao, L. (2006) *Mol. Cancer Res.* **4,** 150–158
17. Su, B., Zheng, Q., Vaughan, M. M., Bu, Y., and Gelman, I. H. (2006) *Cancer Res.* **66,** 5599–5607
18. Streb, J. W., Kitchen, C. M., Gelman, I. H., and Miano, J. M. (2004) *J. Biol. Chem.* **279,** 56014–56023
19. Camus, A., Mesbah, K., Rallu, M., Babinet, C., and Barra, J. (2001) *Mech. Dev.* **105,** 79–91
20. Erlichman, J., Gutierrez-Juarez, R., Zucker, S., Mei, X. H., and Orr, G. A. (1999) *Eur. J. Biochem.* **263,** 797–805
21. Grove, B. D., and Bruchey, A. K. (2001) *J. Vasc. Res.* **38,** 163–175
22. Tao, J., Shumay, E., McLaughlin, S., Wang, H. Y., and Malbon, C. C. (2006) *J. Biol. Chem.* **281,** 23932–23944
23. Ory, D. S., Neugeboren, B. A., and Mulligan, R. C. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 11400–11406
24. Schreiber, E., Matthias, P., Muller, M. M., and Schaffner, W. (1989) *Nucleic Acids Res.* **17,** 6419
25. Lin, X., Nelson, P. J., Frankfort, B., Tombler, E., Johnson, R., and Gelman, I. H. (1995) *Mol. Cell. Biol.* **15,** 2754–2762
26. Papoulas, O., Williams, N. G., and Kingston, R. E. (1992) *J. Biol. Chem.* **267,** 10470–10480
27. Gregor, P. D., Sawadogo, M., and Roeder, R. G. (1990) *Genes Dev.* **4,** 1730–1740
28. Black, A. R., Black, J. D., and Azizkhan-Clifford, J. (2001) *J. Cell. Physiol.* **188,** 143–160
29. Hagen, G., Muller, S., Beato, M., and Suske, G. (1994) *EMBO J.* **13,** 3843–3851
30. Hasleton, M. D., Ibbitt, J. C., and Hurst, H. C. (2003) *Biochem. J.* **373,** 925–932
31. Sapetschnig, A., Rischitor, G., Braun, H., Doll, A., Schergaut, M., Melchior, F., and Suske, G. (2002) *EMBO J.* **21,** 5206–5215
32. Mori, Y., Cai, K., Cheng, Y., Wang, S., Paun, B., Hamilton, J. P., Jin, Z., Sato, F., Berki, A. T., Kan, T., Ito, T., Mantzur, C., Abraham, J. M., and Meltzer, S. J. (2006) *Gastroenterology* **131,** 797–808

33. Tost, J., Schatz, P., Schuster, M., Berlin, K., and Gut, I. G. (2003) *Nucleic Acids Res.* **31,** e50
34. Rombouts, K., Knittel, T., Machesky, L., Braet, F., Wielant, A., Hellemans, K., De Bleser, P., Gelman, I., Ramadori, G., and Geerts, A. (2002) *J. Hepatol.* **37,** 788–796
35. Turner, B. M. (2000) *BioEssays* **22,** 836–845
36. Wu, J., and Grunstein, M. (2000) *Trends Biochem. Sci.* **25,** 619–623
37. Streb, J. W., and Miano, J. M. (2005) *J. Biol. Chem.* **280,** 4125–4134
38. Won, J., Yim, J., and Kim, T. K. (2002) *J. Biol. Chem.* **277,** 38230–38238
39. Choi, H. S., Lee, J. H., Park, J. G., and Lee, Y. I. (2002) *Biochem. Biophys. Res. Commun.* **296,** 1005–1012
40. Doetzlhofer, A., Rotheneder, H., Lagger, G., Koranda, M., Kurtev, V., Brosch, G., Wintersberger, E., and Seiser, C. (1999) *Mol. Cell. Biol.* **19,** 5504–5511
41. Cha, H. J., Okada, A., Kim, K. W., Sato, H., and Seiki, M. (2001) *Clin. Exp. Metastasis* **18,** 675–681
42. Birchenall-Roberts, M. C., Ruscetti, F. W., Kasper, J., Lee, H.-D., Friedman, R., Geiser, A., Sporn, M. B., Roberts, A. B., and Kim, S.-J. (1990) *Mol. Cell. Biol.* **10,** 4978–4983
43. Fujii, M., Shalloway, D., and Verma, I. M. (1989) *Mol. Cell. Biol.* **9,** 2493–2499
44. Qureshi, S. A., Rim, M. H., Alexandropoulos, K., Berg, K., Sukhatme, V. P., and Foster, D. A. (1992) *Oncogene* **7,** 121–125
45. Sugano, S., Stoeckle, M. Y., and Hanafusa, H. (1987) *Cell* **49,** 321–328
46. Sung, J., Turner, J., McCarthy, S., Enkemann, S., Li, C. G., Yan, P., Huang, T., and Yeatman, T. J. (2005) *Carcinogenesis* **26,** 487–494
47. Scholz, G., Martinerie, C., Perbal, B., and Hanafusa, H. (1996) *Mol. Cell. Biol.* **16,** 481–486
48. Joseph, C. K., Qureshi, S. A., Wallace, D. J., and Foster, D. A. (1993) *J. Biol. Chem.* **267,** 1327–1330
49. Ozaki, T., and Sakiyama, S. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 2593–2597
50. Pierani, A., Pouponnot, C., and Calothy, G. (1993) *Mol. Cell. Biol.* **13,** 3401–3414
51. Pan, J., Nakanishi, K., Yutsudo, M., Inoue, H., Li, Q., Oka, K., Yoshioka, N., and Hakura, A. (1996) *FEBS Lett.* **383,** 21–25
52. Ozaki, T., and Sakiyama, S. (1994) *Cancer Res.* **54,** 646–648
53. Enomoto, H., Ozaki, T., Takahashi, E., Nomura, N., Tabata, S., Takahashi, H., Ohnuma, N., Tanabe, M., Iwai, J., and Yoshida, H. (1994) *Oncogene* **9,** 2785–2791
54. Provot, S., Pouponnot, C., Lecoq, O., Calothy, G., and Felder-Schmittbuhl, M. P. (2000) *Oncogene* **19,** 4736–4745
55. Chupreta, S., Du, M., Todisco, A., and Merchant, J. L. (2000) *Am. J. Physiol.* **278,** C697–C708
56. Merchant, J. L., Du, M., and Todisco, A. (1999) *Biochem. Biophys. Res. Commun.* **254,** 454–461
57. Safe, S., and Abdelrahim, M. (2005) *Eur. J. Cancer* **41,** 2438–2448
58. Kuo, L., Chang, H. C., Leu, T. H., Maa, M. C., and Hung, W. C. (2006) *J. Cell Physiol.* **207,** 729–734
59. Sawadogo, M. (1988) *J. Biol. Chem.* **263,** 11994–12001
60. Hadjiagapiou, C., Borthakur, A., Dahdal, R. Y., Gill, R. K., Malakooti, J., Ramaswamy, K., and Dudeja, P. K. (2005) *Am. J. Physiol.* **288,** G1118–G1126
61. Gupta, A. K., and Kone, B. C. (2002) *Am. J. Physiol.* **283,** C1065–C1072
62. Yang, X. P., Freeman, L. A., Knapper, C. L., Amar, M. J., Remaley, A., Brewer, H. B., Jr., and Santamarina-Fojo, S. (2002) *J. Lipid Res.* **43,** 297–306
63. Ge, Y., Jensen, T. L., Matherly, L. H., and Taub, J. W. (2003) *J. Biol. Chem.* **278,** 49901–49910
64. Cress, W. D., and Seto, E. (2000) *J. Cell. Physiol.* **184,** 1–16
65. Minucci, S., and Pelicci, P. G. (2006) *Nat. Rev. Cancer* **6,** 38–51
66. Longworth, M. S., and Laimins, L. A. (2006) *Oncogene* **25,** 4495–4500
67. Hsu, M. C., Chang, H. C., and Hung, W. C. (2006) *J. Biol. Chem.* **281,** 4718–4725
68. Huang, Y. C., and Hung, W. C. (2006) *J. Cell. Physiol.* **209,** 363–369
69. Zhao, S., Venkatasubbarao, K., Li, S., and Freeman, J. W. (2003) *Cancer Res.* **63,** 2624–2630
70. Xiao, H., Hasegawa, T., and Isobe, K. (1999) *J. Cell. Biochem.* **73,** 291–302
71. Maehara, K., Uekawa, N., and Isobe, K. (2002) *Biochem. Biophys. Res. Commun.* **295,** 187–192
72. Yokota, T., Matsuzaki, Y., Miyazawa, K., Zindy, F., Roussel, M. F., and Sakai, T. (2004) *Oncogene* **23,** 5340–5349



Supplementary Fig. 1. Representative western blot of the exogenous Sp1, Sp3, PIAS1, SUMO-1, HDAC1 expressed in the transfected cells described in Figs. 8 and 10. 50 mg of cell lysates prepared from the NIH3T3 cells transfected with the indicated expression plasmids were immunoblotted with antibodies specific for Flag tag (A), Sp3 (B), PIAS1 (C), HA tag (D), and HDAC1 (E). GAPDH is shown as a loading control. For the HDAC1 blot (E), only the exogenous HDAC1, which has a slower electrophoretic mobility due to multiple nuclear localization signals and epitope tags, is shown.